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PHYSIOLOGY OF SPOROCYTOPHAGA STRAINS
ISOLATED FROM SOILS

by

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A STUDY OF THE PHYSIOLOGY
of
SPOROCYTOPHAGA STRAINS ISOLATED FROM SOILS

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CLAIM OF ORIGINALITY

The writer makes the following claims of originality and contributions to knowledge.

A method was developed to study the respiration of Sporocytophaga cells in the Warburg respirometer.

High glucose concentrations (4.5 per cent) were inhibitory but not toxic to Sporocytophaga strain 5-80.

A study was made on the effects of amino acids, proteose-peptone, urea and growth factors on the metabolism of Sporocytophaga strains.

INTRODUCTION

Decomposition of cellulose, by the microorganisms in the soil is a part of the carbon cycle in nature. Aerobic microorganisms play an important role in the decomposition of cellulose but the manner in which it is broken down is not known.

The Cytophagas are a group of active cellulose decomposers common in soil and of interest because of distinct morphological and physiological properties. A study of the physiology of this group may furnish information on the mode of cellulose decomposition.

LITERATURE REVIEW

MORPHOLOGY

van Iterson (1904) was the first to demonstrate the aerobic decomposition of cellulose by bacteria. He observed that cellulose fibres were attacked by a slender vigorously motile non-sporeforming rod, Bacillus ferrugineus, and that in the later stages of decomposition the remnants of the fibres were enveloped in a mucilaginous mass of micrococci. van Iterson could not purify his enriched cultures and concluded that the micrococci, though unable by themselves to break down the cellulose, were living in symbiosis with the rod, assisting it in cellulose decomposition.

Merker (1912) isolated from living leaves of Elodea an aerobic coccus, Micrococcus cytophagus, which caused destruction of the cell walls. Cellulose, as filter paper, was decomposed with a transparent vitreous and yellow growth, which microscopic examination revealed as a zooglear mass of egg-shaped cocci. von Gescher (1922) and Löhnis & Lochhead (1923) observed in cellulose enrichment cultures a large coccoid form which often entirely covered the fibres as a "Mikrokokkenschleim". The above workers had isolated strains of a microorganism

now known as Sporocytophaga. Hutchinson & Clayton (1919), first demonstrated the distinct morphological and cultural properties of this organism and named it Spirochaeta cytophaga. They found that young cultures consisted of long thin flexuous cells; in older cultures a large coccus or ovoid form was invariably present. They could not separate the rod and coccus forms by the dilution method. A dilution series was prepared from what appeared microscopically to be a pure culture of the rod form; growth occurred in a dilution of 10^7 and microscopic examination revealed both thread and coccus forms. They concluded "That in view of the fact that the inoculant used for this set of cultures contained apparently a pure thread form and could most certainly not have contained anything approaching the number of coccus forms indicated by this second dilution set, viz, 40×10^6 per cc., the conclusion was unavoidable that there existed a vital and intimate connection between the two forms". They suggested that the coccus form was a stage in the life cycle of the organism. Microscopic examination of the culture demonstrated intermediate forms in the rod-coccus-life cycle. "The predominating form in young cultures consisted of simple sinuous filaments. With increasing age differentiation of the cell structure

occurred. Cells became progressively shorter and staining showed changes in the chromatin material. The terminals of the cell were less intensely stained while the cell contents assumed the form of a densely stained equatorial band and took up the spherical form. The nuclear substance took up a transverse position and finally divided, this was accompanied by a constriction of the cell wall. The two half cells each contained a disc of nuclear substance which remained attached to the cell wall when the spherical or ovoid stage was reached. Finally the microcyst became evenly and intensely stained, presumably owing to a dispersion of the nuclear substance. The sporoid germinated to produce a vegetative cell". In older cultures the sporoid stage was accentuated. After two to three weeks the whole of the bacterial mass and also of the partially decomposed fibres consisted entirely of micrococci. The lack of flagella and perfect flexibility of the cell suggested a relationship to the Spirochaetoideae. Hutchinson and Clayton, therefore, proposed the name, Spirochaeta cytophaga, recognizing that the organism possessed properties which were not observed in the spirochaetes. The ovoid or spherical form was referred to "for convenience" as a sporoid stage until more information respecting the affinity of this to other organisms was obtained. The changes are diagrammatically shown in the following photograph from Hutchinson & Clayton. (Fig.1)

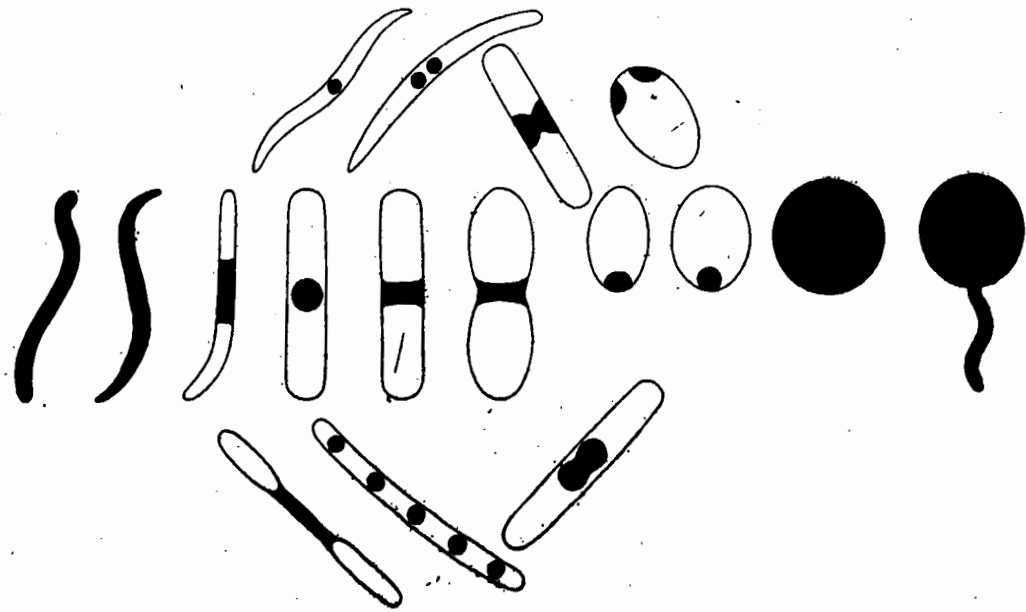


Fig. 1.

Figure 1. Life cycle of Spirochaeta cytophaga from
Hutchinson & Clayton, 1919.

Winogradsky (1929) questioned the purity of cultures obtained by the dilution method. He isolated fresh cultures on cellulose silica gel and showed that colonies could be obtained which contained only the filamentous form. Winogradsky disregarded all colonies which contained the spherical form and thus obtained wholly vegetative Cytophaga cultures. He postulated that the sporoids were contaminants which grew abundantly in the media and were especially evident when the filamentous cell was undergoing autolysis. He regarded the transition stages of the vegetative cell as autolyzed forms. The cycle proposed by Hutchinson and Clayton was interpreted in the following manner: normal multiplication took place only when filamentous Cytophaga cells were in contact with non-decomposed cellulose fibres. The autolytic process developed before the fibre was completely decomposed. Filamentous cells stained poorly, the chromatin material gradually disappeared until finally the filament increased in size, either throughout its whole length or at one of the extremities resulting in elongated, pyriform or tadpole-shaped corpuscles. Corpuscles stained lightly and some contained chromatic granules which later disappeared. The colorless, structureless spheres remained in this state for weeks before disappearing completely. Winogradsky never observed germination of "corpuscles".

He recognized that this organism did not possess any of the properties of the Spirochaetes and, therefore, proposed a new generic name Cytophaga for the group. Type species Cytophaga hutchinsonii which was regarded as identical with Spirochaeta cytophaga isolated by Hutchinson & Clayton. Function strictly specific, cellulose sole source of carbon. No attempt was made to determine the exact position of the genus Cytophaga in bacterial classification.

Krzemieniewska (1930, 1933) confirmed the life-cycle as proposed by Hutchinson & Clayton. She demonstrated the presence of two closely related Cytophaga strains which differed in the nature of the spherical cell. Cytophaga hutchinsonii (Winogradsky) formed non-germinating spherical cells, while Spirochaeta cytophaga (Hutchinson & Clayton) possessed sporoids which germinated to produce the vegetative cell. She suggested that the sporoid was a form analagous to the microcysts of the myxobacteria and therefore, regarded the sporoids as microcysts. The microcysts were surrounded by a mucoid sheath, which on germination disappeared from one side and a cell emerged which elongated to form the vegetative cell. The life cycle appeared similar to that of the myxobacterium Myxococcus, therefore, she proposed the name Cytophaga myxococcoides, in the genus Cytophaga as defined by Winogradsky (1929).

Winogradsky (1932) at first criticized Krzemieniewska's findings, but finally (1935) accepted them. The developmental cycle has been confirmed by a number of workers. Stapp & Bortels (1934) observed germination of microcysts in about twelve hours. Imsenecki & Solntzeva (1936) isolated a new species with oval germinating microcysts. The latter considered the Cytophaga a form of Myxococcaceae which had lost the ability to form sporangia under laboratory conditions. These four authors showed that locomotion among the soil Cytophagas was of the creeping type characteristic of myxobacteria.

Grace (1951) interpreted the chromatic changes as a nuclear development. She stated that "The haploid microcyst germinates, the resulting cell containing two paired chromosomes reproducing vegetatively. An autogamous fusion and subsequent reduction division precede the maturation of the microcyst". A diagrammatic representation of the life cycle was presented but no photomicrographs. Her interpretation of the life cycle was as follows (Fig. 2 & 3):

Germination of the microcyst (Grace 1951)

"The microcyst germinated in approximately 8 hours. The central mass enlarged and was transformed into a thick central bar.

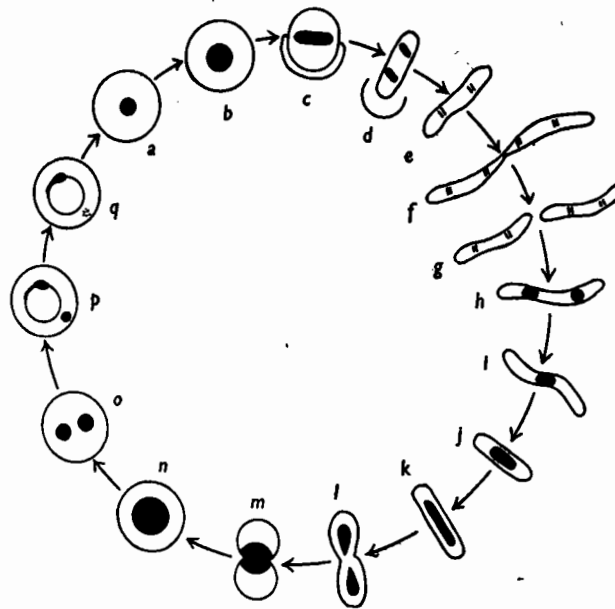


Fig. 1. Diagrammatic representation of the life cycle of *Sporocytophaga*. a, mature microcyst; b-d, germination of the microcyst; e-g, vegetative reproduction; h-q, microcyst formation.

Figure 2. Life cycle of Sporocytophaga from Grace, 1951.

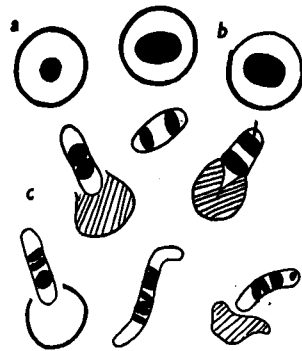


Fig. 2

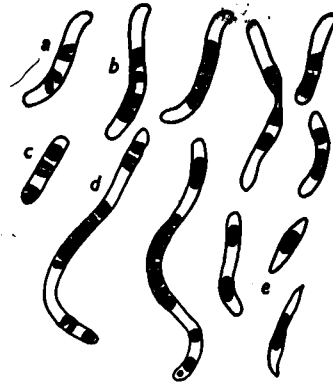


Fig. 3

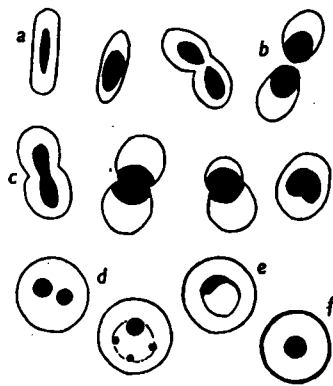


Fig. 4

Figs. 2-4. Drawings from Giemsa-stained preparations of cells of *Sporocytophaga*. Fig. 2. Germinating microcysts of *Sporocytophaga*. a, mature microcyst; b, first stage in the germination of the microcyst; c, liberation of the vegetative cell from the microcyst envelope. Fig. 3. Vegetative cells of *Sporocytophaga*. a, vegetative cell; b, stages in normal vegetative reproduction; c, trinucleate cell; d, filamentous cells; e, first stage in microcyst formation. Fig. 4. Stages in microcyst formation of *Sporocytophaga*. a, b, behaviour of chromatin material preceding fusion; c, nuclear fusion; d, reduction division; e, vesicular nucleus with tadpole-shaped chromatinic body; f, mature microcyst.

Figure 3. Life cycle of Sporocytophaga from Grace, 1951.

The cell shrank slightly, elongated and the chromatin bar divided. The envelope of the cell split and was discarded; as the cell elongated the chromatin bars migrated towards the poles of the cells and were transformed into paired chromatinic bodies characteristic of the vegetative cell.

Vegetative reproduction

Vegetative cells were weakly refractile rods with blunt rounded ends. Ordinary vegetative reproduction was by fission. The four chromosomes divided longitudinally, whilst the cell elongated and constricted at the centre until two daughter cells were formed, each of which contained two pairs of chromosomes.

Microcyst formation occurred after 2 to 8 days. The cells became shorter and the chromosomes were transformed into a central chromatic mass. The whole cell then enlarged slightly and the chromatinic material elongated and divided. The process was accompanied by a central constriction of the cell which sometimes progressed to such

an extent that the two daughter cells were formed. Chromatin bodies fused again at the centre of the constricted cell which became spherical. Fusion nucleus usually divided in two but occasionally four bodies of equal size were formed. One, or in the latter case three of these bodies gradually disappeared. The remaining one was transformed into a large vesicular nucleus. This chromatinic material contracted into a small central body characteristic of the mature microcyst. The outer layer of the cell thickened during the latter stage of microcyst formation and began to stain deeply".

CLASSIFICATION

The genus Cytophaga, as described by Winogradsky (1929), had a strict nutritional requirement, cellulose being the sole source of carbon. Stanier (1941, 1942) demonstrated that the carbon requirements of the Cytophagas were not as restricted as that observed by all previous workers. He proposed a classification which was accepted by the Bergey Committee (Breed et al, 1948). The Cytophagas were classified in two separate families in the order Myxobacteriales.

"Family I Cytophagaceae Stanier

Flexible, sometimes pointed rods, showing creeping motility. No fruiting bodies or spores (microcysts) formed. There is a single genus. Cytophaga.

Genus I Cytophaga Winogradsky

Type species Cytophaga hutchinsonii Winogradsky

Family V Myxococcaceae Jahn

Diagnosis. The rods become shortened when fruiting occurs (resting cells are formed) and develop into spherical or ellipsoidal spores or microcysts. Upon germination the vegetative cell develops from the spore by a process analogous to budding, pinching off at the point of emergence, leaving the spore wall entirely empty. In three of the genera, definite fruiting bodies are produced. In Sporocytophaga, the spores (microcysts) are produced from vegetative cells without development of fruiting bodies .

Genus IV Sporocytophaga Stanier

Diagnosis. Spherical or ellipsoidal microcysts formed loosely in masses of slime among the vegetative cells. Fruiting bodies absent.

Type species Sporocytophaga myxococcoides (Krzemieniewska, Stanier) (Spirochaeta cytophaga, Hutchinson & Clayton)".

The occurrence of microcysts was not considered sufficiently important to warrant separation of the Cytophagas into two separate families. Strains have been reported to have lost the ability to form microcysts (Stanier, 1942, Gray) but unfortunately in both cases the organism died before rigorous tests could be made.

Imsenecki & Solntzeva (1945) suggested a family Promyxobacteriaceae defined as containing myxobacteria which fail to form fruiting bodies, and divided into three genera, Promyxobacterium, Cytophaga and Sporocytophaga. The new genus Promyxobacterium was created for two species which differed slightly in cell length from Cytophaga. Stanier (1947) considered that while it was advantageous to include Cytophaga and Sporocytophaga in one family, the differences observed by Imsenecki & Solntzeva were not distinct enough. Cytophaga johnsonae and C. columnaris contained long or short cells depending on cultural conditions.

Soriano (1945) grouped all the flexible bacteria incapable of forming sporangia into a new order, Flexibacteriales, reserving the order Myxobacteriales for fruiting myxobacteria. The order contained three families:-

1. Flexibacteriaceae

Forms which do not contain granules of sulfur and are incapable of attacking cellulose.

2. Cytophagaceae

Flexible bacteria, spindle-shaped at some point in their development, may or may not produce microcysts, attack cellulose

Genera - Cytophaga and Sporocytophaga

3. Beggiatoaceae

Flexible, filamentous forms, without photosynthetic pigments. Sulfur granules contained in the cells. Does not attack cellulose.

Stanier (1947) criticized this classification "In that the order Flexibacteriales was not in itself a natural assemblage, since the families Flexibacteriaceae and Cytophagaceae differ radically in structure and organization from the Beggiatoaceae".

Tchan et al (1948) proposed a new order Asporangiales, Myxobacteria which never form sporangia. One family, Cytophagaceae, with three genera Sporocytophaga, Cytophaga and Flexibacter. The genera Sporocytophaga and Cytophaga were similar to Stanier's. The genus Flexibacter (Soriano) was a provisional genus which permitted the classification of Myxobacteria still poorly defined.

Bisset (1952) suggested a new order Flexibacteriales, bacteria with "muscular" cell walls, sub-order Myxobacteriineae, unicellular short bacteria. Family, Cytophagaceae, do not form fruiting bodies. Genera, Cytophaga, Sporocytophaga, Chitinoclasta.

NITROGEN METABOLISM

Hutchinson & Clayton (1919) showed that the nitrogen requirement of Spirochaeta cytophaga was supplied by a number of mineral nitrogen compounds. Peptone was utilized as a nitrogen source up to a concentration of 0.1 per cent but could not act as the sole nutrient. Stronger solutions, 0.25 per cent, had an inhibitory action on the growth of the organism.

Bucksteeg (1936) compared various nitrogen sources for a Cytophaga strain, in the presence and absence of calcium carbonate. The experiment showed that the unfavorable effect of NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ was probably due to a lowering of the pH of the medium on the absorption of the ammonium ion. Hutchinson & Clayton (1919) claimed that with increased abstraction of the nitrogen from sodium nitrate, the reaction of the solution would be better maintained owing to the formation of sodium carbonate or of neutral salts of acid by-products.

Reese (1947) confirmed the observation that ammonium

and nitrate acted as good sources of nitrogen for the organism and demonstrated the relationship between the anion and the pH effect. He showed that the optimal concentration of nitrogen for cellulose decomposition by Sporocytophaga myxococcoides was between 1.0 to 2.0 grams NaNO_3 per litre.

Stanier (1942) demonstrated that the following substances were suitable nitrogen sources for the soil Cytophagas.

<u>Substance</u>	<u>S. myxococcoides</u>	<u>C. hutchinsonii</u>	<u>C. rubra</u>
Control - N	0	0	0
$(\text{NH}_4)_2\text{SO}_4$ 0.1%	4	4	4
$(\text{NH}_4)_2\text{SO}_4$ 1.0%	0	0	0
KNO_3 0.1%	4	4	4
KNO_3 1.0%	2-3	0	0
Glycine 0.1%	0	0	0
Alanine 0.1%	0	0	0
Leucine 0.1%	0	0	0
Aspartic acid 0.1%	1	3	3
Aspartic acid 0.5%	3	4	4
Asparagin 0.1%	2-3	2	2
Asparagin 1.0%	3-4	2	4
Peptone 0.1%	4	4	4
Peptone 1.0%	2	2	2
Yeast autolysate 1.0%	1-2	3	3
Urea 0.1%	2-3	0	0

0 - no growth 4 - maximum growth

The monoamino and monocarboxylic amino acids, (glycine, alanine, leucine) were not utilized, whereas both aspartic acid and asparagin provided good growth. Peptone 1.0 per cent and yeast autolysate were good nitrogen sources.

Hutchinson & Clayton (1919) noted that urea (carbamide) had an inhibitory effect on cellulose decomposition, growth occurring in cellulose salts medium only after 40 days. This inhibitory effect of urea was confirmed by Reese who showed that when urea was added to a basal medium containing sodium nitrate, the rate of cellulose decomposition was considerably reduced. A significant decrease of cellulose decomposition was obtained with .002 M urea (.056 grams per litre).

Jensen (1940), Fuller & Norman (1943) showed that NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, asparagin, sodium asparaginate, yeast extract and peptone were suitable nitrogen sources for the cellulose decomposing soil Cytophagas. Imsenecki & Solntzeva (1936) claimed that weak development of Cytophaga was possible in media without nitrogen other than that present as traces of organic nitrogen in the cellulose.

A C:N ratio (cellulose decomposed to nitrogen assimilated) of 30:1 was reported by Hutchinson & Clayton (1919). Jensen (1931) obtained a value of 47:1. The following table from Jensen illustrates representative C:N ratios for cellulose decomposing bacteria and fungi.

Ratio of cellulose decomposed to nitrogen assimilated
(Jensen, 1931)

Bacteria	Ratio	Fungi	Ratio
<u>Vibrio napi</u>	30:1	<u>Coccospora</u> sp.....	30:1
<u>Vibrio prima</u>	33:1	<u>Monosporium</u> sp.....	25:1
<u>Spirochaeta cytophaga</u> ..	47:1	<u>Mycogone nigra</u>	54:1
<u>Vibrio</u> sp.....	43:1	<u>Botryosporium</u> sp....	48:1
		<u>Stachybotrys</u> sp.....	49:1

The nitrogen requirements of the noncellulose decomposing Cytophagas were more complex. Stanier (1940) isolated the marine agar digesting strains, Cytophaga diffluens and Cytophaga krzemieniewskae which utilized peptone and yeast extract as sole sources of nitrogen. Cytophaga columnaris, a myxobacterium pathogenic for fish (Garnjobst, 1945) grew satisfactorily in a medium containing peptone or hydrolyzed casein. Growth was absent in glucose mineral salts medium plus KNO_3 or $(\text{NH}_4)_2\text{SO}_4$. Pure cultures of Cytophaga sensitiva (Humm, 1946) were obtained only on agar media to which were added 0.1 per cent peptone, 0.05 per cent beef extract, 0.5 per cent glucose and traces of yeast extract and ferric phosphate. The optimum peptone concentration was 0.1 per cent; growth was inhibited by concentrations of peptone exceeding 0.4 per cent. The optimum nitrate concentration was 0.5 per cent.

Stanier (1947) isolated a chitin decomposing myxobacterium, Cytophaga johnsonae. Nitrate, ammonia, alanine and peptone

served as sources of nitrogen. Development in glucose salts medium was accelerated by the addition of small amounts of peptone or yeast extract. Maximum development occurred on complex nitrogenous media; Difco tryptone was found to be particularly favorable. In general, concentrations of peptone and other proteinaceous materials of 1.0 per cent or over are deleterious and when development in such media occurred, viability turned out to be poor. Peptone had a marked effect on colony formation. At concentrations of 1.0 per cent or over the primary development was always compact and raised with an entire edge. At less than 1.0 per cent swarming was more evident and 0.5 per cent produced colonies with a slightly raised centre and a broad flat actively "motile periphery". At concentration of 0.25 per cent or less completely flat, rapidly spreading almost invisible swarms were produced.

Sinden, Mix & Siu (1948) observed that the fungus Gliomastix convoluta required an organic source of nitrogen for cellulose decomposition. Yeast extract supported cellulolytic activity, but the effect was not due to thiamin, riboflavin, pantothenic acid, inositol or biotin. Experiments showed that a low concentration of organic nitrogen was required for good growth. The main factor in yeast extract was apparently an organic nitrogen compound.

The nitrogen metabolism of Cytophaga strains was thus shown to range from simple nitrogenous compounds to more complex forms.

CARBON NUTRITION

Hutchinson & Clayton (1919) were the first to study the metabolism of the Cytophagas. They demonstrated an apparent "Obligate cellulose decomposition"; cellulose was the only carbon compound with which growth was obtained. The organism failed to grow on the higher alcohols, carbohydrates and organic acids. Compounds which possessed reducing properties inhibited cellulose decomposition, even in low concentrations, e.g. 0.018 per cent maltose and 0.05 per cent dextrose. Their results are summarized in Table 1.

Winogradsky (1929), Krzemieniewska (1930), Stapp & Bortels (1934), Imsenecki & Solntzeva (1936), Bucksteeg (1936), Walker & Warren (1938), Jensen (1940) all confirmed the observation of Hutchinson & Clayton that cellulose was the only carbon source utilized by Cytophaga.

Imsenecki (1936) showed that the toxic effect of glucose was not connected with its reducing properties. A glucose concentration of 0.5 per cent was inhibitory but growth occurred at oxidation reduction potentials lower than that which resulted from the addition of this concentration

Table I

The relative effects of carbohydrates and organic acids
on growth of Spirochaeta cytophaga (Hutchinson & Clayton, 1919).

	Growth after 12 days			
	Without cellulose		with cellulose	
	0.1%	1.0%	0.1%	1.0%
Mannite	-	-	+	+
Dulcite	-	-	+	+
Adonite	-	-	+	+
Arabinose	-	-	-	-
Dextrose	-	-	-	-
Levulose	-	-	-	-
Galactose	-	-	-	-
Saccharose	-	-	+	+
Maltose	-	-	-	-
Lactose	-	-	-+	-
Raffinose	-	-	+	+
Starch	-	-	+	+
Dextrin	-	-	+	+
Inulin	-	-	+	+
Calcium formate	-	-	+	-
Calcium acetate	-	-	+	-
Calcium oxalate	-	-	++	-
Calcium propionate	-	-	-	-
Calcium butyrate	-	-	-	-
Calcium malate	-	-	++	-
Calcium tartrate	-	-	++	+
Calcium citrate	-	-	++	++

of glucose to the cellulose salts medium. He also showed that the toxic effect was not lethal but merely growth-inhibiting. Stanier isolated non cellulose decomposing marine Cytophagas (1940) with a less restricted nutrient requirement. He re-examined the carbon nutrition of the soil forms and obtained results in agreement with those of the earlier investigators. However, when Stanier (1942) examined the effect of reducing sugars on the respiration of the organisms, the results were entirely unexpected. The oxygen uptake of Cytophaga hutchinsonii growing on cellulose, cellobiose and glucose was measured in a Warburg apparatus. Auto-respiration was high but glucose and cellobiose caused an increase of oxygen uptake comparable with that from cellulose. Thus cellobiose and glucose, like cellulose, were used by the bacteria in an oxidative process. Stanier concluded that the inhibitory effect of glucose was not due to its reducing powers but was caused by sterilization of the sugar in an alkaline medium in the presence of phosphate. The growth experiments were repeated with sugar solution sterilized by filtration through Seitz filters and then added to the autoclaved mineral base. Good growth of the soil Cytophaga strains was obtained over a wide range of glucose concentrations. The filtered glucose exerted no toxic effect on growth in the presence of cellulose as was found in earlier work where

autoclaved glucose was used. High glucose concentrations decreased cellulose decomposition but growth was observed in the surrounding liquid medium. Stanier (1942) suggested this partial inhibition was probably due to preferential utilization of the monosaccharides. The carbon requirements were tested with Seitz filtered sugars. Sporocytophaga myxococcoides and Cytophaga hutchinsonii utilized cellobiose and glucose in addition to cellulose. Cytophaga rubra grew in cellobiose, glucose, mannose and xylose. The optimum glucose concentration varied with different strains as shown in Table II.

Stanier observed that growth on glucose agar medium was somewhat different. Sporocytophaga myxococcoides and Cytophaga rubra developed regularly with glucose at 0.2 per cent, but irregularly at 1.0 per cent concentration.

Fahraeus (1941) working independently also showed that Cytophaga globulosa and a newly isolated strain 3 were able to utilize glucose and cellobiose provided small concentrations of sugar were used. Quantitative determinations of glucose and cellulose showed that glucose, added in low concentrations (0.05 per cent) to the cellulose medium, was rapidly consumed and that the inhibition of cellulose was due to a preferential attack on the glucose. Higher glucose concentrations (0.1 per cent for Cytophaga globulosa, 0.25 per cent for strain 3) inhibited growth for about ten days

Table II

Growth of S. myxococcoides, C. hutchinsonii and C. rubra
with varying concentrations of glucose after 5 days
at 30°C (Stanier, 1942)

Organism	Glucose, per cent						
	0.05	0.1	0.25	0.5	0.75	1.0	2.0
<u>S. myxococcoides</u>	3	3	4	4	4	4	4
<u>C. hutchinsonii</u>	2	2	3	4	4	4	3
<u>C. rubra</u>	2	3	4	3	3	3	3

0 - no growth

4 - maximum growth

after which glucose was consumed first and then the cellulose.

These results were confirmed by Harmsen (1946) who showed that small quantities of soluble sugars stimulated development of the Cytophagas and that only concentrations of more than 0.1 per cent were injurious. Harmsen did not, however, obtain growth in the absence of cellulose.

Fahraeus (1947) further examined the glucose and cellulose metabolism of Cytophaga strain 3 and Cytophaga globulosa. In qualitative experiments, sugars were autoclaved separately and added aseptically to the medium. Inhibition of cellulose decomposition was obtained by concentrations of glucose greater than 0.1 per cent. Cytophaga strain 3 grew on glucose, mannose and cellobiose but not in arabinose, xylose, fructose, galactose, maltose, sucrose, raffinose, dextrin or starch. In quantitative experiments, the residual cellulose was determined and glucose determinations were made by the method of Somogyi (1937). The effects of increasing amounts of glucose on cellulose decomposition by Cytophaga 3 were studied. The results indicated that in all cases glucose was preferentially utilized as a source of carbon. The cellulose was not attacked until most of the glucose had been consumed. When the glucose was completely used up cellulose decomposition proceeded at the same rate as in the flasks without any addition of glucose. High concentrations of glucose (0.25 and 0.5 per cent) had

an inhibitory effect. When the inhibition was overcome the rate of cellulose decomposition was as high as that obtained at lower glucose concentrations. The results are summarized in Table III.

When glucose (250 mg and 100 mg) was added to flasks in which cellulose decomposition was taking place, the decomposition of cellulose was stopped immediately and the glucose was attacked instead of the cellulose. The glucose inhibition was less apparent, probably due to the larger number of bacteria present. Fahraeus (1947) suggested that the checking of the decomposition process was connected with the absence of extracellular cellulase in the medium. Utilization of glucose inhibited cellulase production which was not formed again until the concentration of glucose falls below a certain level, in these experiments. The results are shown in Table IV.

Siu (1951) suggested that the cellulose-sparing action of simple sugars parallels the findings of Kendall & Walker (1915) in connection with the utilization of proteins by Proteus vulgaris. Gelatinase was inhibited by glucose in low concentrations, inhibition continued until the carbohydrate was completely utilized. Concentrations in excess of 0.3 per cent produced a permanent inhibition. Sijpesteijn & Fahraeus (1949) showed that Seitz filtered as well as separately autoclaved glucose was inhibitory in relatively

Table III

Cellulose and glucose decomposition in cultures of Cytophaga 3 (Fahraeus, 1947)

Original cellulose 503 mg									
Glucose added per flask, mg	0	50		100		250		500	
	cell- ulose	cell- ulose	glu- cose	cell- ulose	glu- cose	cell- ulose	glu- cose	cell- ulose	glu- cose
10 days									
Cellulose and glucose consumed, mg	56	37	50	14	68	0	48	0	24
20 days									
Cellulose and glucose consumed, mg	105	82	50	48	100	4	199	5	181
30 days									
Cellulose and glucose consumed, mg	138	116	50	91	100	24	250	15	253

Table IV

Cellulose and glucose decomposition in cultures of Cytophaga 3 and C. globulosa (Fahraeus, 1947).

Strain	Original cellulose 481 mg							
	<u>Cytophaga 3</u>				<u>C. globulosa</u>			
Glucose added per flask, mg	0		250		0		100	
	cell- ulose	glu- cose	cell- ulose	glu- cose	cell- ulose	glu- cose	cell- ulose	glu- cose
10 days								
Cellulose consumed	90				91			
14 days								
Cellulose & glu- cose consumed	134		93	70	142		118	72
20 days								
Cellulose & glu- cose consumed	184		91	118	199		152	98
28 days								
Cellulose & glu- cose consumed	241		93	186	266		211	97

high glucose concentrations (0.5 and 1.0 per cent). Stanier (1942), however, showed that Seitz filtered glucose permitted maximal growth in glucose concentrations up to 2.0 per cent and exerted no "toxic" effect on cellulose decomposition.

The Cytophaga strains (Sijpesteijn & Fahraeus, 1949) grew in mannose but not in fructose and xylose. Cells grown on 1.0 per cent glucose developed without delay in a medium containing 1.0 per cent mannose. Adaptation to a particular high glucose concentration was observed on continued transfer in media with the same high glucose concentration (0.5 and 1.0 per cent); growth occurred after 3 days, in a shorter time than that observed in experiments in which very heavy inocula from cellulose cultures were used. The "toxic" effect of heat sterilization of glucose in alkaline medium was confirmed. However, when glucose was autoclaved in the medium at pH 7.0 the results were comparable with those obtained in media to which separately sterilized glucose was added.

Growth on glucose agar was irregular. Sijpesteijn & Fahraeus (1949) reported that, "It should be stressed that development on glucose agar was not always successful; in several cases growth failed completely notwithstanding that under the same conditions in other instances good growth had been obtained".

A group of non-cellulose specific Cytophagas have been isolated from soil, sea water and fish (dermal parasite). These organisms utilized a wide variety of organic compounds as a source of carbon. The carbon nutrition is summarized in Table V.

MINERAL REQUIREMENTS

Hutchinson & Clayton (1919) observed good growth of Cytophaga in a physiological saline solution containing PO_4 , Mg, Ca, NaCl, Fe and N; concentrations varied slightly but no significant differences of growth were noted (Stanier, 1942, Fahraeus, 1947). Fahraeus (1947) showed that Ca and Mn strongly stimulated growth of Cytophaga strain 3 on cotton wool but had no effect with filter paper. Reese (1947) demonstrated that $MgSO_4$, 0.5 g/litre, doubled the rate of cellulose decomposition by Sporocytophaga myxococcoides and showed that Fe salts were stimulatory but when PO_4 concentration was increased from 0.01 M to 0.06 M the stimulatory effect of Fe disappeared.

OXYGEN REQUIREMENTS

The Cytophagas are strictly aerobic, growth occurs only at the air-water interface in tubes with paper strips. Imsenecki & Solntzeva (1936) showed that cellulose decomposition

Table V

Carbon nutrition of Cytophaga strains (Breed et al, 1948)

Carbon Source	<u>Cytophaga</u> strains						
	1	2	3	4	5	6	7
Glucose	+	+	+	+	+	+	-
Fructose							
Galactose	-	+	+	+	+	+	
Mannose						+	
Maltose		+	+	+	+	+	
Lactose	+	+	+	+	+	+	
Sucrose		+	-	-	+	+	
Cellobiose			+	+		+	
Raffinose							
Starch		+	+		+	+	-
Inulin						+	
Cellulose	+		+	+		-	-
Cellulose dextrin	+	+			+		
Gum arabic		+					
Pectin		+			+		
Agar-agar		+	+				
Alginic acid		+	+				
Chitin			-	-		+	
Hemi cellulose	+	+			+		
Xylose			+	+		+	
Arabinose			-	-	+	+	
Calcium gluconate					+		
Proteinaceous materials					+	+	+

- Strain 1 Cytophaga deprimata (Fuller & Norman, 1943)
 2 C.albogilva (Fuller & Norman, 1943)
 3 C.krziemieniewska (Stanier, 1940)
 4 C.diffluens (Stanier, 1940)
 5 Sporocytophaga congregata (Fuller & Norman, 1943)
 6 C.johnsonae (Stanier, 1947)
 7 C.columnaris (Garnjobst, 1945)

was increased from 10 per cent without aeration to 46 per cent with aeration in a 10-day incubation period. The stimulatory effect of added oxygen or air for increased cellulose decomposition was confirmed by Walker & Warren (1938), Norman & Bartholomew (1940) and Fahraeus (1947). Reese (1947) found that shaking (100 cycles per minute) increased fourfold the rate of cellulose decomposition by Sporocytophaga myxococcoides. Imsenecki & Solntzeva (1936) stated that Cytophaga was capable of decomposing cellulose with a limited supply of oxygen but this has not been confirmed by other workers (Jensen, 1940 and Stanier, 1942).

GROWTH FACTOR REQUIREMENTS

The effect of growth factors on the rate of cellulose decomposition by Cytophaga strains has not been extensively investigated. Stanier (1942) showed that 1.0 per cent yeast autolysate was a good nitrogen source for soil Cytophaga strains. Reese (1947) found that no increase in cellulose decomposition by Sporocytophaga myxococcoides was obtained on addition of yeast extract to the mineral salts medium. Fahraeus (1947) demonstrated that Cytophaga 3 formed considerable quantities of thiamin in a mineral salts medium; cellulose decomposition was obvious after 3 days in the presence of 0.1 gamma thiamin, but only after 6 days in the controls without thiamin, and then not in all tubes.

The increased rate of cellulose decomposition in the presence of thiamin was not observed when the experiment was repeated; Fahraeus suggested that possibly the inoculum was too large and thus sufficient quantities of thiamin were added with the inoculum.

Stapp & Bortels (1934), Imsenecki & Solntzeva (1936), and Harmsen (1946) found that associated contaminating bacteria had a stimulating effect on growth and cellulose decomposition by Cytophaga strains. Stanier (1942), Harmsen (1946) suggested that accompanying bacteria stimulated growth and cellulose decomposition in a strictly mineral salts medium with cellulose, by providing growth factors in amounts necessary for maximal growth.

pH

The soil Cytophagas show the greatest cellulose decomposing activity at a neutral or slightly alkaline pH. Growth occurred at the pH range of 6.0 to 8.5, while pH values of 5.0 and 9.0 were inhibitory (Walker & Warren, 1938, Fahraeus, 1947, Reese, 1947, and Stapp & Bortels, 1934). Fahraeus (1947) showed that the decomposition of cellulose resulted in a fall in pH as shown in Table VI. At first growth occurred most rapidly at pH range 7.4 to 7.7, development at pH 7.0 was slower while growth was negligible at 8.3. After 30 days greatest decomposition occurred in the series pH 8.3 to 7.7. The flask which was originally adjusted to pH 8.3 was now at the optimum pH.

Table VI

pH experiment with Cytophaga 3 (Fahraeus, 1947)

Original cellulose 517 mg						
pH range	8.3	7.7	7.4	7.0	6.4	5.8
7 days						
Cellulose consumed	2	62	62	53	22	-
Final pH	8.0	7.3	7.1	6.8	6.4	
30 days						
Cellulose consumed	180	185	174	162	143	117
Final pH	7.3	7.1	6.9	6.6	6.0	5.9 to 5.6

TEMPERATURE

Temperature for growth

Several workers found that the optimum temperature range for cellulose decomposition by Cytophaga strains was 28° to 30°C (Imsenecki & Solntzeva, 1936, Jensen, 1940, Stanier, 1942 and Fahraeus, 1947). Sijpesteijn & Fahraeus (1949) observed that the lag period of growth in 0.5 and 1.0 per cent glucose was diminished by incubation at 25°C.

The temperature for killing Sporocytophaga myxococcoides was reported by (1) Hutchinson & Clayton (1919) (Spirochaeta cytophaga) as 62°C exposure for 5 minutes or 58°C for 10 minutes; (2) by Krzemieniewska (1930) as 62°C for the microcysts and 58°C for the vegetative cells and (3) by Imsenecki & Solntzeva as 68°C. Sijpesteijn & Fahraeus purified cultures of Sporocytophaga strain D by "pasteurization" of contaminated cultures at 68° to 70°C.

Temperature and motility

Movement of the soil Cytophagas was relatively rapid at 28° to 30°C, motility was inhibited at lower temperatures and was only slight at 20°C (Stapp & Bortels, 1934, Imsenecki & Solntzeva, 1936).

Temperature and development

Stanier (1942) observed that microcysts were formed most abundantly at 30° to 35°C while at 20°C the process

could be entirely suppressed. "Strains maintained continuously at room temperature may not form microcysts for months at a time but when placed at a higher temperature the process becomes established again".

BIOCHEMISTRY OF CELLULOSE DECOMPOSITION

Pringsheim (1912) demonstrated the accumulation of reducing sugars when growth of a vigorous thermophilic cellulose decomposer was inhibited by antiseptics (sisting method). The reducing sugars were identified as cellobiose and glucose. He postulated that cellulose was decomposed by two hydrolytic enzymes, cellulase and cellobiase. Pringsheim's results were confirmed by other workers (Simola, 1931, Kalnins, 1930, Imsenecki, 1936 and Jensen, 1940) for different strains of bacteria and fungi.

Siu (1951) suggested a hydrolytic decomposition of cellulose by a series of enzymes resulting in the production of cellobiose or glucose. Whitaker (1951) concentrated and purified the cellulase of Myrothecium verrucaria and showed that the cellulase possessed a distinct electrophoretic pattern as obtained by electrophoresis on filter paper.

Products

Hutchinson & Clayton (1919) showed that when cellulose was decomposed by Spirochaeta cytophaga, a mucilage was formed

together with small amounts of butyric acid and pigment, but no reducing substances. Winogradsky (1929) considered the Cytophagas as highly specialized cellulose decomposers. Since he could not demonstrate the presence of reducing sugars, he postulated that terminal carbinol groups on the cellulose molecule were oxidized to aldehydic groups and that these and even other alcohol groups might further be oxidized to carboxyl groups. The evidence was qualitative, based on the finding that decomposed filter paper was acidic, stained intensely with the basic dye, methylene blue, and was soluble in alkaline solutions from which it could be precipitated with acid. The properties were considered similar to those of chemically produced oxycellulose. Quantitative analyses of the mucilage (Walker & Warren, 1938, Loicjanskaja, 1937) demonstrated the presence of uronic acid units. This was considered as additional evidence that the initial stage of cellulose breakdown involved oxidation of the cellulose into a polyglucuronic acid. Norman & Bartholomew (1940) Fahraeus (1944, 1947) confirmed the polyglucuronide nature of the mucilage but they suggested that it was a product of bacterial metabolism rather than oxycellulose.

Norman & Fuller (1942) stated that "An oxidative attack presupposes the existence of an oxidative exo-enzyme system. The postulated product, oxycellulose, is water insoluble and

would still be unavailable to the organism. No energy could be derived by the cell until sufficient chain splitting had occurred to render the fragments water-soluble, and if this could take place, there would seem to be no obvious reason why an insoluble oxycellulose should accumulate". They suggested that the process of cellulose decomposition was probably hydrolytic.

Fahraeus (1944, 1947) showed that a mucilage of similar chemical properties was obtained from Cytophaga cultures grown in cellulose or in glucose. He suggested the following course of cellulose decomposition



Cytophaga hydrolyzed the cellulose to glucose which was absorbed by the cell as a source of carbon for respiration and synthesis.

Reducing substances

Fahraeus (1944) demonstrated that enzyme preparations from Cytophaga strain 3 yielded reducing substances from hydrated cellulose (Table VII). The culture flasks contained 10 mg of dried bacteria, 5 ml M/30 phosphate buffer and 58 mg hydrated cellulose (Scales method). Cellulose and glucose determinations were made after 24 hours.

Table VII

Consumption of cellulose and formation of reducing substances by enzymes from Cytophaga strain 3 (Fahraeus, 1944).

Results are expressed as mg of glucose per sample of 5 ml		
	reducing substance mg	cellulose mg
Control	2.0	60.0
Sample with enzyme	9.7	49.4
Difference	7.7	10.6

Sixty-five per cent of the cellulose consumed was recovered as reducing substances ("Calculated as glucose, 10.6 mg cellulose give theoretically 11.8 mg glucose"). The reducing substances had an optical rotation of 52.5° , an osazone crystal structure and melting point identical with that of glucose. Fahraeus concluded that "By these results it may be regarded as fully established that the reducing substance that arose out of cellulose with Cytophaga enzymes is identical with glucose".

Carbon dioxide

Walker & Warren (1938), Bokor (1930), Stanier (1942) demonstrated that the main end product of cellulose decomposition by Cytophaga is carbon dioxide.

Acid production

Hutchinson & Clayton (1919) observed that a volatile acid, presumably butyric acid was formed in cultures of Spirochaeta cytophaga. Later investigations (Walker & Warren, 1938, Jensen, 1940, Norman & Bartholomew, 1940, Stanier, 1942) failed to show any acid formation in Cytophaga cultures. Fahraeus (1949) consistently found a certain amount of volatile acid in flask cultures. The kind of acid was not definitely established but "A recent qualitative examination by means of the adsorption technique of Elsdon (1946) indicates that mainly acetic acid and in addition small

amounts of propionic acid are formed in cultures of two different strains of Cytophaga" (Fahraeus, 1949).

Pigment

Walker & Warren (1938) claimed that "The yellow pigment formed by Cytophaga hutchinsonii is considered to be an unsaturated aliphatic acid, of relatively small molecular dimensions and is not as stated in the literature (Hutchinson & Clayton, 1919) a carotenoid pigment."

RESPIRATION

The resting cell technique has been widely used in studies of bacterial respiratory enzymes. Wilson (1938) discussed the problems associated with the preparation of bacterial resting cells. He stressed the following:

- (a) the organisms should be readily separable from the suspending or washing solutions;
- (b) the cells should possess a high respiration rate;
- (c) the endogenous metabolism should not be excessive;
- (d) the rate of respiration should be directly proportional to the number of viable organisms present;
- (e) the activity of the suspension should be stable.

The application of this method for the study of Cytophaga respiration was difficult because copious slime formation made it impossible to separate the cells from the mucilage.

Stanier (1942) utilized 3 day old cellulose grown cultures which were lightly centrifuged to remove unattacked particles of cellulose. He showed that oxygen uptake by Cytophaga hutchinsonii was increased in the presence of either glucose or cellobiose in the medium. The endogenous respiration was high.

Fahraeus (1946, 1947) obtained Cytophaga enzyme preparations by precipitation of the mucilage with 20 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ or with acid. The culture liquid was concentrated in vacuo after dialysis. He showed that the rate of cellophane decomposition by Cytophaga enzymes was decreased in the presence of glucose, however, a "Considerable hydrolysis took place" in the presence of 0.5 per cent glucose. The enzyme preparations hydrolyzed cellobiose but had no effect on soluble starch, dextran, yeast mannan, salicin, raffinose, trehalose, maltose, lactose and sucrose.

The purpose of the present work, in the main, was to study:

- (a) The effects of carbohydrates, nitrogen compounds, yeast extract and growth factors on the rate of cellulose decomposition by Cytophaga strains.
- (b) The effects of organic compounds on the endogenous and glucose respiration of "resting" Cytophaga cells as measured in the Warburg apparatus.

This required:

- 1 Isolation of the bacteria from soil and manure.
- 2 Purification and classification of the isolated strains.
- 3 An investigation of the relationship between the vegetative and microcyst forms.

METHODS

ISOLATION AND PURIFICATION

Isolation

Sporocytophaga strains were isolated by the following methods:

Omelianski's method (Omelianski, 1895)

(i) A filter paper strip was partially immersed in basal medium, in a five-eighth inch tube. The medium was inoculated with a soil particle or with 1 ml. of a 1:10 or 1:100 dilution of a soil suspension. After 3 to 5 days incubation at 25° to 30°C the filter paper became discoloured at or slightly above the level of the liquid. A shred of filter paper was picked off and subcultured into a sterile tube of the same medium. When growth appeared in this tube a microscopic examination was made for the typical Cytophaga cells and microcysts. When present, subcultures were made until a highly enriched culture was obtained. If absent, two further subcultures were made before the soil was regarded as "Cytophaga negative".

(ii) A variation of the above method was to fold a filter paper in such a way that when it was placed in a 125 ml conical flask it spread out "tent-wise", thus providing a larger surface area; 25 ml of basal medium was added to the

flask, which was then autoclaved at 15 lbs pressure for 15 minutes. The procedures that followed were similar to those under (i) above.

Winogradsky's method (Winogradsky, 1929)

A circle of filter paper 4" in diameter was placed on the surface of mineral agar in a Petri plate. The plate was inoculated at regular intervals with small particles of soil. The method of inoculation was as follows:

(i) A finely drawn out glass rod, with flattened tip, was used to deposit small soil particles at regular intervals on the plate. This method was adopted for manure and moist soil samples.

(ii) For dry soils, the above method was modified as follows: a Gooch crucible was used to sprinkle soil on the surface of the filter paper. The cellulose decomposers developed around the soil particles. Microscopic examination was made for Cytophaga cells; when these were present a shred of attacked filter paper was inoculated into a tube of cellulose salts medium.

Soil plate method

Twenty grams of soil at 60 per cent water holding capacity were placed in a Petri plate and covered with a 4" circle of filter paper. After 4 to 7 days incubation, discoloured shreds of filter paper were examined microscopically

for the presence of Cytophaga cells. When present, the procedure was as outlined above.

Pure culture methods

The highly enriched cultures were purified in the following ways:

Dilution method

A dilution series from 1:10 to 1:10⁷ was prepared with the enriched culture as the primary inoculum. After 5 to 7 days a second dilution series was inoculated from the highest positive dilution. The first series was followed for 14 days; on first evidence of growth in a higher dilution than that from which series 2 was prepared, a further dilution series was made. The object was to obtain an inoculum of cells before marked cellulose decomposition had occurred and the contaminants were at a minimum. The procedure was continued until a pure culture was obtained. The purity test was lack of growth in beef extract broth combined with a microscopic examination.

Heating methods

The microcysts were more resistant than the non-sporeforming contaminants to high temperatures. The enriched cultures were heated for 10 minutes at 58°C and 65°C. Subcultures were made at 5 minutes to cellulose salts medium; at 10 minutes the culture was brought rapidly

to room temperature by immersion in tap water, and a dilution series was prepared. The highest dilution showing growth was plated on glucose agar.

Plating method

(i) A filter paper strip was placed on the surface of mineral salts agar Petri plate. The paper was inoculated by streaking a loopful of organisms from a highly enriched culture. After 5 to 7 days isolated yellow spots developed. Attacked paper from these spots was removed and emulsified in a few ml of basal medium. The emulsion was diluted 1:10 and 1:100 and used as the inoculum for a second plating series. The process was repeated until a pure culture was obtained.

Regenerated cellulose

(ii) Regenerated cellulose was used instead of filter paper. The regenerated cellulose was prepared by Kalnins' modification of Scales' method (Kalnins, 1930); by dissolving filter paper in Schweitzer's solution (Kellerman & McBeth, 1912); or in cold sulphuric acid (Fuller & Norman, 1942); the methods are described on page 122 in the appendix.

Glucose agar

(iii) The Sporocytophaga strains used in the following experiments were obtained from isolated colonies on glucose agar. The enriched cultures were plated on 0.1 per cent

glucose washed agar. Generally 2 plates were used, the second being inoculated with a higher dilution of cells. The inoculum was the emulsion of a few strands of attacked filter paper diluted 1:10 and 1: 100. Isolated colonies were picked off and inoculated into tubes of cellulose salts medium. These cultures were then replated, reisolated and kept in tubes of cellulose salts liquid media as stock cultures.

MICROSCOPIC EXAMINATION

The organisms were examined in the living state by means of wet mounts. Morphological examinations were also made by staining the cells. The staining procedure was as follows:

A few shreds of attacked filter paper were placed in a drop of water on a clean slide. The preparation was air dried, fixed by gentle heat and stained for 30 seconds with crystal violet. The crystal violet solution was prepared by dissolving 1 gm in 10 ml of 95 per cent ethyl alcohol and diluting the solution with 90 ml of distilled water.

MEDIA

The basal medium was prepared by dissolving the following inorganic salts in distilled water:

	Medium	
	A %	B %
K_2HPO_4	0.1	0.1
$MgSO_4 \cdot 7 H_2O$	0.02	0.05
NaCl	0.02	0.02
$CaCl_2$	0.01	0.01
$NaNO_3$	0.05	-
NH_4NO_3	-	0.05
$FeCl_3 \cdot 6 H_2O$	0.002	-
$FeSO_4 \cdot 12 H_2O$	-	0.002

The first five salts were prepared in 10 times concentration and ferric chloride in 2 per cent concentration, and diluted to give the desired strength. Glucose, cellulose, or other nutrients were added to the basal medium. Unless otherwise stated the pH of the medium was adjusted to 7.1 to 7.2 with N/1 NaOH or N/1 HCl.

The glucose broth was basal medium with 0.1 per cent glucose. The glucose solution was sterilized by filtration through a Seitz filter, by autoclaving in distilled water, or

in the basal medium at pH 6.8.

Glucose agar: 1 ml of 1.0 per cent sterile glucose solution was mixed in a Petri plate with 10 ml of basal salts washed agar (1.0 per cent) to give the required 0.1 per cent concentration of glucose.

Cellulose broth: a strip of filter paper, Whatman Nos. 41, 42 or 44 was immersed so as partly to project above the surface of the basal salts medium in a culture tube.

CARBON COMPOUNDS

The test sugar was added in a concentration of 0.1 per cent to the basal medium, the pH was adjusted with N/1 HCl to 6.8. Nine ml of the test medium was added to a 5/8 tube containing a strip of Whatman filter paper. The tubes were sterilized at 10 lbs pressure for 15 minutes. The sterility of the medium was checked by incubating for 3 days at 30°C. Duplicate tubes were inoculated with a loopful of attacked fibres from a 7 day old culture of the test organism. All cultures showing growth after 10 days were inoculated into cellulose basal medium and checked for purity by lack of growth in beef extract broth.

The following carbohydrates were tested as carbon sources for the Cytophaga strains:

A. Monosaccharides

D-Glucose C.P.	E & A
D-Fructose C.P.	B.D.H.
D-Galactose C.P.	Fisher Scientific Co.
D-Mannose C.P.	EKCO

B. Disaccharides

Maltose	B.D.H.
Lactose	Difco
Sucrose	E & A
Cellobiose	EKCO

C. Trisaccharides

D-Raffinose hydrate	N.B.
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D. Polysaccharides

1. Starch group

Starch	Difco soluble starch
Inulin C.P.	Pfanstiehl Chem. Co.
Dextrin, pure, precipitated by alcohol	Baird and Tatlock

2. Cellulose group

Cellulose	Whatman No. 42
Hemi-cellulose	

Pentosan: Gum arabic E & A
(Acacia)

Hexosan: Agar-agar	Rose & Laflamme
Hexo-pentosan: Pectin, citrus	Research Dept. California Fruit Growers Exchange

3. Pentoses

Arabinose C.P.	Fisher
Xylose	EKCO

Eimer & Amend	E & A
British Drug Houses	B.D.H.
Eastman Kodak Co.	EKCO
Nutritional Biochem. Co.	N.B.

The results of these tests are shown in Table VIII.

NITROGEN COMPOUNDS

Sources of inorganic nitrogen:

$(\text{NH}_4)_2\text{HPO}_4$	B.D.H.
NH_4NO_3	B.D.H.
KNO_3	E & A
NaNO_3	Merck
NH_4SO_2	Merck
$(\text{NH}_4)_2\text{SO}_4$	Merck
Ammonium tartrate	Merck
Ammonium oxalate	Merck

Each of the above compounds was added to the basal salts medium in a concentration of 0.05 per cent.

The following amino acids were tested in cultural and

Warburg experiments:

Source

dl-alpha Alanine	EKCO
l-Arginine monohydrochloride	Merck
Asparagine	E & A
dl-Aspartic acid	EKCO
l-Cysteine hydrochloride	EKCO
d-Glutamic acid	EKCO
Glycine	EKCO
dl-Leucine	Merck
dl-Lysine	EKCO
dl-Phenylalanine	Brickman
l-Proline	N.B.
dl-Tryptophan	Brickman
Tyrosine	Merck
dl-Valine	EKCO

The following amino acids were only tested in the Warburg experiment:

l-Glutamic acid	dl-Norleucine
l-Histidine monohydrochloride	dl-Ornithine
Hydroxy-l-proline	dl-Serine
dl-Isoleucine	l-Taurine
dl-Methionine	dl-Threonine

Source Nutritional Biochem. Co.

The amino acid requirement was determined by growing the organisms in the presence of each individual amino acid as the sole nitrogen source. The effects of amino acids were further investigated by growing the organisms in a mixture from which amino acids of similar chemical structure were omitted. The effect of the grouping was compared to media containing nitrate, yeast extract and yeast extract with nitrate.

The combinations of amino acids in these media were such that those having related characteristics were omitted from the 14 amino acid combination, as shown below (Wallace & Lochhead, 1950).

14 amino acid combination alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, leucine, lysine, phenylalanine, proline, tryptophan, tyrosine, valine.

Grouping	Amino acids omitted from 14 amino acid combination
1	alanine, glycine
2	cysteine
3	leucine, valine
4	aspartic acid, glutamic acid
5	arginine, asparagine, lysine
6	proline
7	phenylalanine, tryptophan, tyrosine

A 2.5 per cent concentrated stock solution of each amino acid was prepared. In preparation of the mixtures 1 ml of the required amino acid was added to 50 ml of double concentration basal medium. When all the amino acids were added the volume of the test medium was made up to 100 ml with distilled water. Each amino acid was thus present in the test medium at a concentration of 0.025 per cent.

The individual amino acids were tested in a 0.25 per cent concentration, 1 ml of the concentrated solution was added to 99 ml of basal medium.

Yeast extract and sodium nitrate were added when required at a concentration of 0.025 per cent and 0.05 per cent respectively.

The pH of each medium was adjusted (Beckman pH meter) to pH 6.9 to 7.1 with N/1 NaOH or N/1 HCl.

Nine ml of test medium was added to a strip of Whatman filter paper in a 5/8 inch test tube. Tubes were inoculated (triplicate) with a 5 mm loopful of suspension of attacked cellulose fibres from 7 day old Cytophaga cultures.

The results are shown in Tables IX to XV.

GROWTH FACTORS

The growth factors studied were, calcium pantothenate, biotin, thiamin hydrochloride, nicotinic acid, pyridoxine hydrochloride and riboflavin; and were assigned a code number from 1 to 6 respectively.

A stock solution was prepared for each growth factor.

The concentrated stock solution was diluted 1 in 10 with distilled water; 1 ml of the test growth factor was then added to 50 ml of double concentrated basal medium, when all growth factors were added the volume was adjusted to 100 ml with distilled water. Final concentration for each growth factor was 1 $\mu\text{g/ml}$ except biotin which was present in a final concentration of 1 mg/ml .

Growth factor	Source	Code no.	Concentration of stock solution	Final concentration
Calcium pantothenate	Merck	1	1 mg/ml	1 $\mu\text{g/ml}$
Biotin	N.B.	2	10 $\mu\text{g/ml}$	1 mg/ml
Thiamin hydrochloride	Merck	3	1 mg/ml	1 $\mu\text{g/ml}$
Nicotinic acid	N.B.	4	1 mg/ml	1 $\mu\text{g/ml}$
Pyridoxine hydrochloride	Merck	5	1 mg/ml	1 $\mu\text{g/ml}$
Riboflavin	EKCO	6	1 mg/ml	1 $\mu\text{g/ml}$

Duplicate tubes of basal cellulose medium, plus test growth factor were inoculated with a 5 mm loopful of washed cells.

A growth factor free inoculum was prepared by centrifuging 6 day old Cytophaga cultures grown in glucose basal medium. The cells were washed three times (under aseptic conditions) by centrifuging, decanting the supernatant and then resuspending the cells in basal salts medium. Sterility was indicated by lack of growth in beef extract broth.

Clean glassware was soaked in the detergent, Calgonite, for half hour, rinsed 6 times in hot tap water followed by 10 rinsings in distilled water.

Test media included all possible combinations of growth factors. The 63 media were arranged in 21 series as follows:

<u>Series 1</u>	<u>Series 2</u>	<u>Series 3</u>	<u>Series 4</u>	<u>Series 5</u>
a 34561	a 45612	a 56123	a 61234	a 12345
b 4561	b 5612	b 6123	b 1234	b 2345
c 561	c 612	c 123	c 234	c 345
d 61	d 12	d 23	d 34	d 45
e 1	e 2	e 3	e 4	e 5

<u>Series 6</u>	<u>Series 7</u>	<u>Series 8</u>	<u>Series 9</u>	<u>Series 10</u>
a 23456	a 1345	a 5214	a 1235	a 1246
b 3456	b 134	b 514	b 135	b 246
c 456	c 13	c 14	c 15	c 46
d 56				
e 6				

<u>Series 11</u>	<u>Series 12</u>	<u>Series 13</u>	<u>Series 14</u>	<u>Series 15</u>
a 2456	a 3256	a 4326	a 1356	a 4136
b 245	b 256	b 326	b 356	b 136
c 24	c 25	c 26	c 35	c 36

<u>Series 16</u>	<u>Series 17</u>	<u>Series 18</u>	<u>Series 19</u>	<u>Series 20</u>
123456	124	125	235	346

Series 21

146

The results of these experiments are shown in Table XVI and Appendix Tables XXX to XXXVI.

Yeast extract

Yeast extract concentrations were prepared by dilution from a 1.0 per cent concentration, with basal medium as diluent as follows:

	Volume of basal medium in ml	Yeast extract concentration %
3 grams yeast extract	300	1.0
104 ml of 1.0%	156	0.4
104 ml of 0.4%	104	0.2
104 ml of 0.2%	104	0.1
104 ml of 0.1%	104	0.05
104 ml of 0.05%	104	0.025
13 ml of 0.05%	117	0.005
0 ml control	100	0.000

The test medium was dispensed in 10 ml quantities in tubes containing strips of Whatman filter paper. Test media were inoculated with either a 5 mm loopful of attacked cellulose fibres or washed cells.

RESPIRATION STUDIES

Oxygen uptake was measured in a Warburg apparatus at 30°C, with air as the gas phase. Carbon dioxide produced was absorbed by filter paper, saturated with 10 per cent KOH, in the centre well of the Warburg flask.

The Warburg flask contained the following substances:

	0.5 ml of test substance
Main chamber	1.0 or 2.5 ml of cell suspension
	1.0 ml M/30 PO_4 buffer pH 7.2
	nitrogen free basal medium.
Centre well	0.2 ml 10 per cent KOH.

Final volume of the main chamber was 3.0 ml. Test substance and cell suspension were diluted in nitrogen free basal medium B.

M/10 PO_4 buffer 17.8 gm $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, plus approximately 20 ml N/1 HCl, diluted to 1 litre, the pH was adjusted to 7.2 with a Beckman pH meter; final concentration was M/30.

Amino acids Stock solution 1.5 per cent was diluted 1:10; 0.5 ml was added to the main chamber, final concentration was 0.025 per cent.

Carbohydrates Glucose, mannose, fructose, galactose, 0.054 gm to 10 ml; 0.5 ml was added to the main chamber, final concentration 0.09 per cent (M/200);

Carbohydrates	cellobiose, lactose, sucrose final concentration M/400; starch, inulin final concentration 0.1 per cent; cellulose (Scales hydro-cellulose) final concentration 0.1 per cent.
Peptone	Proteose-peptone Difco final concentration 0.1 per cent.
Yeast extract	Difco final concentration 0.05 per cent.
Urea	Final concentration 0.1 per cent.
pH	Phosphate buffer was adjusted to pH 5.9, 6.5, 7.2, 8.5 with N/1 NaOH or N/1 HCl.
Basal medium	A 3 litre, double concentration, stock solution of medium B less nitrogen was prepared. Nitrogen source was 0.05 per cent NH_4NO_3 .

Cell suspensions

The cell suspensions were obtained by the following methods:

Agar

Agar surfaces in Roux flasks and dilution bottles, were inoculated with a 1 ml suspension of Cytophaga cells grown in glucose yeast extract broth. When growth occurred the culture was scraped off and suspended in 25 ml of nitrogen free basal medium.

Glucose mass culture

Three litre bell flasks, each containing 2 litres of basal medium with 0.1 per cent Seitz filtered glucose and 0.05 per cent yeast extract were inoculated with 1 ml of a 4 day old culture of strain 5-80 grown in glucose broth. The culture was harvested after 3 to 5 days with the Sharples supercentrifuge and the cells were suspended in 25 ml of nitrogen free basal medium.

Centrifugation

Cultures grown in glucose yeast extract broth were centrifuged at 7,000 R.P.M. for 2 to 3 hours.

The methods described above were not satisfactory and the following method was finally adopted.

Candle filtration

One litre flasks with 200 ml basal medium were inoculated with 1 ml Seitz filtered glucose-yeast extract at a final concentration of 0.1 per cent glucose and 0.05 per cent yeast extract. The flasks were incubated for 3 days to insure sterility. Sterile flasks were inoculated with shreds of attacked filter paper from cellulose strips in tubes of basal medium, and shaken at approximately 60 c.p.m. for 60 to 70 hours. As the incubation temperature varied and the inoculum was not constant, flasks were removed when good growth was visible but before copious slime formation occurred.

Cells were concentrated by filtering the suspension through sterile candle filters and washed on the candle with sterile nitrogen-free basal medium. The film of cells was easily floated off the surface of the candle into 25 ml of sterile nitrogen-free basal medium in a large culture tube. The suspension was centrifuged under aseptic conditions to remove small particles of filter paper. The bacteria were used immediately or stored overnight at 10°C. Preparations were made daily. The method was simple and active cell suspensions were obtained. The sterility of all flasks was determined by microscopic examination and by inoculations from random culture flasks into beef extract broth and cellulose salts medium (Figure 3a).

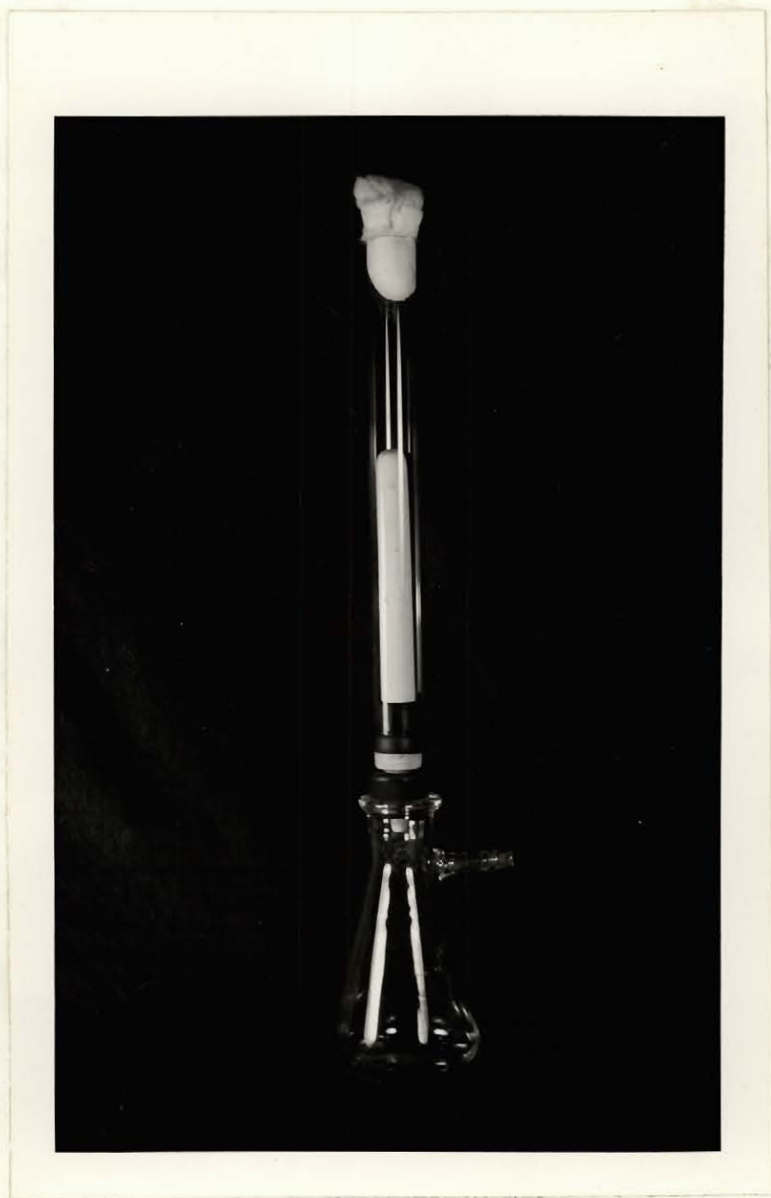


Figure 3a. Candle filtration.

RESULTS

Sources and description of isolated strains

Soil samples from the Macdonald College area were examined by the enrichment method for the presence of Cytophagas. Samples were taken from forest soil, cultivated land, a strawberry plot under straw cover, fresh and old manure piles. The results are summarized in Table VII A

Table VII A

Occurrence of Cytophaga in soil samples from Macdonald College area.

Source	No. of samples	No. showing cellulose decomposition	No. containing <u>Cytophaga</u>	Strains studied
Forest soil	16	16	1	-
Cultivated soils	9	9	0	-
Strawberry plot	9	9	5	5-80
Manure	10	10	7	B-1
Swampy field	1	1	1	XXB

The bacteria were readily isolated from the strawberry plot and manure but not from the cultivated and forest soils. The previous treatment of the cultivated soils was not ascertained. A pure culture of Sporocytophaga myxococcoides from the American Type Culture Collection, No. 10010 was also studied, coded as X-20.

Description of the isolated strains:

Sporocytophaga strains	Filamentous cells		Spherical microcysts
	width μ	length μ	μ
5-80	0.3 to 0.5	2.5 to 7.0	1.0 to 1.3
XXB	0.3 to 0.5	2.0 to 7.0	1.0 to 1.3
B-1	0.5	2.0 to 7.0	1.0 to 1.3
ATCC 10010	0.3 to 0.5	2.5 to 7.0	1.0 to 1.3

DILUTION EXPERIMENT

The object of this experiment was to investigate the relationship between the microcyst and vegetative stages.

A four day old culture of Sporocytophaga myxococcoides, American Type Culture Collection No.10010, in cellulose broth was the primary inoculum which was subcultured at 2, 3, 4, and 6 day intervals. The inoculum was a loopful of the undisturbed medium. Each subculture was resubcultured at similar time intervals. All positive cultures were examined for presence of microcysts. The subculture series was coded. The numeral 1 represented the primary inoculum; the numbers 2, 3, 4, 5, 6 etc. indicated subculture time intervals. For example, 1233 showed that the primary inoculum was subcultured after two days (12); three days later culture 12 was subcultured (123); three days after this 123 was again subcultured (1233).

Growth and microcysts were obtained in the series underlined in the following Flow Sheets.

No growth was obtained with a 2 day subculture interval (12222); thus cultures 12 and 122 were negative but positive growth was obtained in 1224 and 123. No reason can be given to explain why growth occurred with the greater dilution

of cells existing in the two latter series; possibly the inoculum consisted of cells from tube 1 which were still viable in tubes 12 and 122 but were unable to grow.

Two positive subcultures at 3 or 4 day intervals were obtained in series 1-2-3, tubes 1233 and 12334. In series 1-3 growth occurred more consistently at the longer time intervals between subculture.

The 4 and 5 day incubation periods (Series 1-4 and 1-5) yielded more positive cultures. Growth was not obtained consistently at these longer time intervals; for example, 1444 was positive while the following tube 14445 was negative. In only one case did growth occur throughout the series, 135545; however, in the corresponding culture 135455 only 1354 was positive. A separation of the microcyst and vegetative forms was not obtained in these experiments.

Dilution experiment

Flow Sheet No. 1

Series 1-2

Series 1-2-4

Series 1-2-2

1242

12222

1245

1224

Series 1-2-6

12244

12655

1224455

12245

Series 1-2-8

122635

12835

Series 1-2-3

1233

12334

123342

123343

123345

12335

123352

123355

1235

1236

123665

Dilution experiment

Flow Sheet No. 2

Series 1-3

Series 1-3-5

Series 1-3-2

1352

1323

135235

1325

135245

132535

135255

13254

1354

1326

135425

132635

135435

132665

135455

1327

1355

13272

135525

132725

13554

13275

135545

Series 1-3-3

13555

1336

135552

Dilution experiment
Flow Sheet No. 3

Series 1-4

Series 1-4-6

Series 1-4-2

1462

1422

146255

142225

1463

14225

14635

142255

1464

1424

14645

142455

1426

142655

Series 1-4-4

1442

144225

144253

14427

1444

144425

144435

144455

1445

144525

14455

Dilution experiment

Flow Sheet No. 4

Series 1-5

Series 1-5-3

1532

153235

153254

15327

153275

1533

15335

153365

1535

15352

153522

153525

15353

153545

15355

Series 1-5-5

1552

155255

1553

15535

1554

155435

15545

Series 1-5-6

1562

15625

1563

1566

15665

CARBOHYDRATE METABOLISM

All strains utilized glucose, mannose, cellobiose and cellulose. The results are shown in Table VIII.

Table VIII

Growth of Sporocytophaga strains with 0.1 per cent carbohydrate as sole source of carbon.

	Strain			
	X-20	5-80	XXB	B-1
Glucose	3	3	3	3
Fructose	-	-	-	-
Galactose	-	-	-	-
Mannose	3	3	3	3
Maltose	-	-	-	-
Sucrose	-	-	-	-
Cellobiose	3	3	3	3
Raffinose	-	-	-	-
Starch	-	-	-	-
Inulin	-	-	-	-
Dextrin	-	-	-	-
Cellulose	+++	+++	+++	+++
Gum arabic	-	-	-	-
Agar-agar	-	-	-	-
Pectin	-	-	-	-
Xylose	-	-	-	-
Arabinose	-	-	-	-

- no growth 3 maximum growth
+++ vigorous cellulose decomposition

NITROGEN METABOLISM

The effects of inorganic nitrogen on cellulose decomposition are shown in Table IX. The results indicated the suitability of ammonium and nitrate as sources of nitrogen and the inhibitory effect of the associated ion. The oxalate ion completely inhibited growth of strain X-20 and permitted only limited growth of strains XXB and B-1.

Amino acids

The effects of amino acids are shown in Table X. Asparagine was a good nitrogen source for all strains. Limited growth was also obtained with cysteine and glutamic acid. With the remaining amino acids, growth was obtained in arginine, aspartic acid and tyrosine with organism X-20. Spotted growth was obtained with lysine with organisms X-20 and XXB.

The phenomenon of spotted growth was the formation of yellow spots on the filter paper above or below the surface of the liquid and was a characteristic feature of growth with amino acids. The inoculum for these studies was purposely a heavy one and the growth obtained may have been due to the deposition of a small particle of decomposed paper from the inoculum on the surface of the test cellulose strip, in which case growth could probably occur in the immediate vicinity but would otherwise be inhibited by the conditions of the medium.

Table IX

Effect of sources of inorganic nitrogen on
cellulose decomposition after 14 days

Sporocytophaga strains

Nitrogen source	X-20	XXB	B-1
$(\text{NH}_4)_2\text{HPO}_4$	+++	+++	+++
NH_4NO_3	++	++	++
KNO_3	+++	+++	+++
NaNO_3	+++	+++	+++
$(\text{NH}_4)_2\text{SO}_4$	+++	+++	+++
NH_4SO_2	+	+	+
Ammonium oxalate	-	-+	-+
Ammonium tartrate	+	+	+

The degree of decomposition of cellulose is indicated thus:

- lacking
 -+ very faint
 + distinct
 ++ fairly strong
 +++ vigorous

Table X

Effect of amino acids on cellulose decompositon

Sporocytophaga strains

	X-20	5-80	B-1	XXB
Days at which cellulose decompositon was evident				
Basal medium	3 +++	3 +++	3 +++	3 +++
Alanine	-	-	-	-
Arginine	16 +	-	-	-
Asparagine	3 +++	3 ++	3 +++	3 +++
Aspartic acid	10 +	-	-	-
Cysteine	5 +	10 +	5 +	10 +
Glutamic acid	10 ++	28 s	24 +	15 s
Glycine	-	-	-	-
Leucine	-	-	-	-
Lysine	15 s	-	-	15 s
Phenylalanine	-	-	-	-
Proline	-	-	-	-
Tryptophan	-	-	-	-
Tyrosine	10 +	-	-	-
Valine	-	-	-	-
Basal less nitrogen	-	-	-	-

The degree of decomposition of cellulose after 28 days is indicated thus:

-	lacking	++	fairly strong
-+	very faint	+++	vigorous
+	distinct	s	spotted growth

Amino acids with 0.05 per cent NaNO_3

When a readily available nitrogen source was added to the amino acids growth was greatly improved as shown in Table XI. Glycine and leucine, however, had a marked inhibitory effect on the rate of cellulose decomposition. A similar inhibition was also observed with alanine and phenylalanine with strain X-20 and cysteine with strain 5-80.

Table XI

Effects of amino acids with 0.05 per cent NaNO_3 on cellulose decomposition.

Sporocytophaga strains

	X-20	5-80	B-1	XXB
Days at which cellulose decomposition was evident				
Basal medium	4	4	7	3
Alanine	10	4	5	10
Cysteine	7	10	3	3
Glycine	-	15	15	15
Leucine	-	10	15	24
Phenylalanine	10	3	5	5

- no growth in 28 days

Cellulose decomposition was obtained in 3 or 4 days with the other 9 amino acids.

Combinations of amino acids

The effects of combinations of amino acids on the rate of cellulose decomposition are listed in Tables XII to XV. Cellulose decomposition by strain X-20 was almost completely inhibited. Positive and spotted growth was obtained only in media B 2 and D 2 respectively after 28 days; growth was observed only in the controls, where sodium nitrate and yeast extract were sole sources of nitrogen.

The growth of organism 5-80 was greatly retarded in the combinations of amino acids, excepting medium A 7 and A 2 (Table XIII). In medium A 7, where tryptophan, phenylalanine and tyrosine were missing, growth was obtained in 7 days; in medium A 2 (combination less cysteine) growth was observed in all replicates in 15 days. In all other instances growth was irregular, either spotted or positive in one out of the 3 replicates inoculated. The development of the organism was improved when 0.5 per cent NaNO_3 was added to the medium; except medium B 6 (combination less proline) which was negative after 28 days. The addition of yeast extract to the medium had a definite stimulating effect, notably with medium C 6 and C 7.

Table XII

Effect of combinations of amino acids on cellulose decomposition by Sporocytpha strain X-20.

Medium group no.	Treatment			
	A	B	C	D
	Medium only	Medium & 0.05% NaNO ₃	Medium & 0.025% yeast extract	Medium & NaNO ₃ & yeast extract
Days at which cellulose decomposition was evident				
0	-	5	10	5
1	-	-	-	-
2	-	28	-	s
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
All	-	not tested	not tested	-

- no growth in 28 days

Medium
group no.

All Alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, leucine, lysine, phenylalanine, proline, tryptophan, tyrosine, valine.

0 Control, basal medium with cellulose strip
 1 All less alanine, glycine
 2 All less cysteine
 3 All less leucine, valine
 4 All less aspartic acid, glutamic acid
 5 All less arginine, asparagine, lysine
 6 All less proline
 7 All less phenylalanine, tryptophan, tyrosine

Table XIII

Effect of combinations of amino acids on cellulose decomposition by Sporocytophaga strain 5-80.

Medium group no.	Treatment			
	A	B	C	D
	Medium only	Medium & 0.05% NaNO ₃	Medium & 0.025% yeast extract	Medium & NaNO ₃ & yeast extract
Days at which cellulose decomposition was evident				
0	-	3 ++	3 +	5 ++
1	28 s	7 ++	10 +	10 ++
2	15 +	7 +++	15 +	5 ++
3	9 s	10 +++	7 +++	7 ++
4	9 s	7 ++	7 ++	7 ++
5	11 s	15 ++	7 +	10 ++
6	6 s	-	10 ++	24 ++
7	6 ++	5 +++	3 +++	5 ++
All	28 ++	not tested		

Decomposition of cellulose after 28 days is indicated as follows:

-	lacking	++	fairly strong
-+	very faint	+++	vigorous
+	distinct	s	spotted growth

Cellulose decomposition was observed in 6 days with organism XXB in medium A (Table XIV). The other combinations had a definite inhibitory effect. Growth was obtained with medium 5 (combination less arginine, asparagine, lysine) only on addition of 0.025 per cent yeast extract. Yeast extract also had a definite stimulating effect on the development of the organism in medium 4 (combination less aspartic and glutamic acids).

The best development in the combination of amino acids was obtained with organism B-1 (Table XV). In most of the media growth was evident in 6 days. However, growth was obtained in medium 5 only on addition of yeast extract or sodium nitrate to the medium. The addition of sodium nitrate to the media had a beneficial effect on growth of the organism. Yeast extract was stimulatory for media 4 and 7.

Table XIV

Effect of combinations of amino acids on cellulose decomposition by Sporocytophaga strain XXB.

Medium group no.	Treatment			
	A	B	C	D
	Medium only	Medium & 0.05% NaNO ₃	Medium & 0.025% yeast extract	Medium & NaNO ₃ & yeast extract
Days at which cellulose decomposition was evident				
0	-	3 +++	3 +	5 +++
1	15 +	10 +++	5 +++	5 +
2	9 ++	10 +++	10 +	16 +++
3	15 ++	10 +++	10 ++	16 +++
4	15 ++	16 s	7 ++	24 ++
5	-	-	10 +	-
6	15 ++	10 +++	7 +++	16 +++
7	6 ++	5 +++	3 +++	7 +++
All	11 s		not tested	

Decomposition of cellulose after 28 days is indicated as follows:

-	lacking	++	fairly strong
-+	very faint	+++	vigorous
+	distinct	s	spotted growth

Table XV

Effect of combinations of amino acids on cellulose decomposition by Sporocytophaga strain B-1.

Medium group no.	Treatment			
	A	B	C	D
	Medium only	Medium & 0.05% NaNO ₃	Medium & 0.025% yeast extract	Medium & NaNO ₃ & yeast extract
Days at which cellulose decomposition was evident				
0	-	5 ++	10 +	5 ++
1	10 ++	7 +++	7 ++	7 ++
2	9 +++	5 +++	16 ++	7 +++
3	6 ++	5 +++	5 ++	7 +++
4	6 ++	5 +++	3 ++	5 +++
5	-	5 +++	10 ++	5 +++
6	6 +++	5 +++	5 +++	16 ++
7	6 +++	5 +++	3 +++	3 +++
All	5 +++	not tested		

Decomposition of cellulose after 28 days is indicated as follows:

-	lacking	++	fairly strong
-+	very faint	+++	vigorous
+	distinct	s	spotted growth

GROWTH FACTORS

The effects of the growth factors, biotin, pyridoxine, pantothenic acid, nicotinic acid, riboflavin and thiamin, on the rate of cellulose decomposition, are recorded in Appendix Tables XXX to XXXVI. The results are summarized in Table XVI which shows the number of test media and percentage of total test media showing cellulose decomposition at different time intervals.

The greatest stimulation was obtained with strain 5-80, 75 per cent of the test media were positive before growth was visible in the control. Strain X-20 showed 11 per cent positive test media. With strains XXB and B-1, growth occurred in the test media and controls at the same time.

The stimulation could not be attributed to any particular group of growth factors.

Yeast extract

Concentrations of yeast extract from 0.2 per cent to 0.025 per cent stimulated cellulose decomposition of strains 5-80, B-1 and XXB. Organism X-20 showed slight stimulation of cellulose decomposition at a concentration of 0.025 per cent. Concentration of 1.0 per cent inhibited growth of all strains, 0.4 per cent inhibited only strain X-20. The results are listed in Tables XVII and XVIII.

Table XVI

Effects of growth factors on cellulose decomposition by Sporocytophaga strains.

Strains	Time days	No. of media showing cellulose decomposition	Per cent of total media
X-20	4 to 5	7	11
	9*	42	67
	14	51	81
5-80	4 to 5	47	75
	9*	55	87
	14	58	92
XXB	4 to 5*	58	92
	9	62	98
	14	63	96
B-1	4 to 5*	50	80
	9	58	92
	14	60	95

Total number of test media 63

* Growth present in control medium.

Table XVII

The effect of yeast extract on cellulose decomposition by an inoculum of washed cells.

Sporocytophaga strains

	X-20	5-80	XXB	B-1
Yeast extract % (concentration)	Days at which cellulose decomposition was evident			
1.0	-	-	-	-
0.4	19	7	7	7
0.2	10	4	4	7
0.1	7	4	4	7
0.05	7	4	4	7
0.025	7	4	4	7
0.005	7	7	7	7
Basal medium	10	7	7	7

- no growth in 28 days

Table XVIII

The effect of yeast extract on cellulose decomposition by a standard inoculum.

Sporocytophaga strains

	X-20	5-80	XXB	B-1
Yeast extract%				
Days at which cellulose decomposition was evident.				
1.0	-	-	-	-
0.4	19	7	7	4
0.2	7	4	4	4
0.1	7	4	4	4
0.05	7	4	4	4
0.025	4	4	4	4
0.005	7	7	7	4
Basal medium	7	7	7	7

- no growth in 28 days

RESPIRATION STUDIES

Carbohydrate metabolism

Starch, inulin, cellulose, lactose, sucrose and fructose were not oxidized by Sporocytophaga strain 5-80 (Table XX). Oxygen consumption was increased in the presence of glucose, mannose and cellobiose (Table XX, Figures 4, 5). The percentage increase in oxygen uptake above endogenous is shown in Table XIX. Glucose had the greatest effect, followed by mannose and cellobiose.

Table XIX

Percentage increase in oxygen consumption, above endogenous, by Sporocytophaga strain 5-80, in presence of glucose, mannose and cellobiose.

Glucose	Mannose	Glucose	Cellobiose
126.5	107	137	70

The optimum glucose concentration was M/200 (0.09 per cent). Respiration occurred within the range of M/4 (4.5 per cent) to M/1000 (0.018 per cent) (Tables XXI, XXII, XXIII and Figure 6). M/2 (9.0 per cent) glucose inhibited respiration but was not toxic. M/1 (18.0 per cent) glucose inhibited endogenous and glucose respiration. No oxygen uptake occurred at M/10,000 (0.0018 per cent), 18 micrograms per litre.

Table XX

The effect of carbohydrates on oxygen uptake by Sporocytophaga strain 5-80.

Accumulative oxygen uptake in microlitres

Time minutes	Endogenous	Starch	Inulin	Cellulose	Glucose
120	118.5	129.4	117.2	116.7	240.1
	Endogenous	Cellobiose	Lactose	Sucrose	Glucose
120	78.1	132.8	84.2	77.4	185.7
	Endogenous	Mannose	Fructose	Galactose	Glucose
150	91.5	189.4	98.2	92.9	208.2

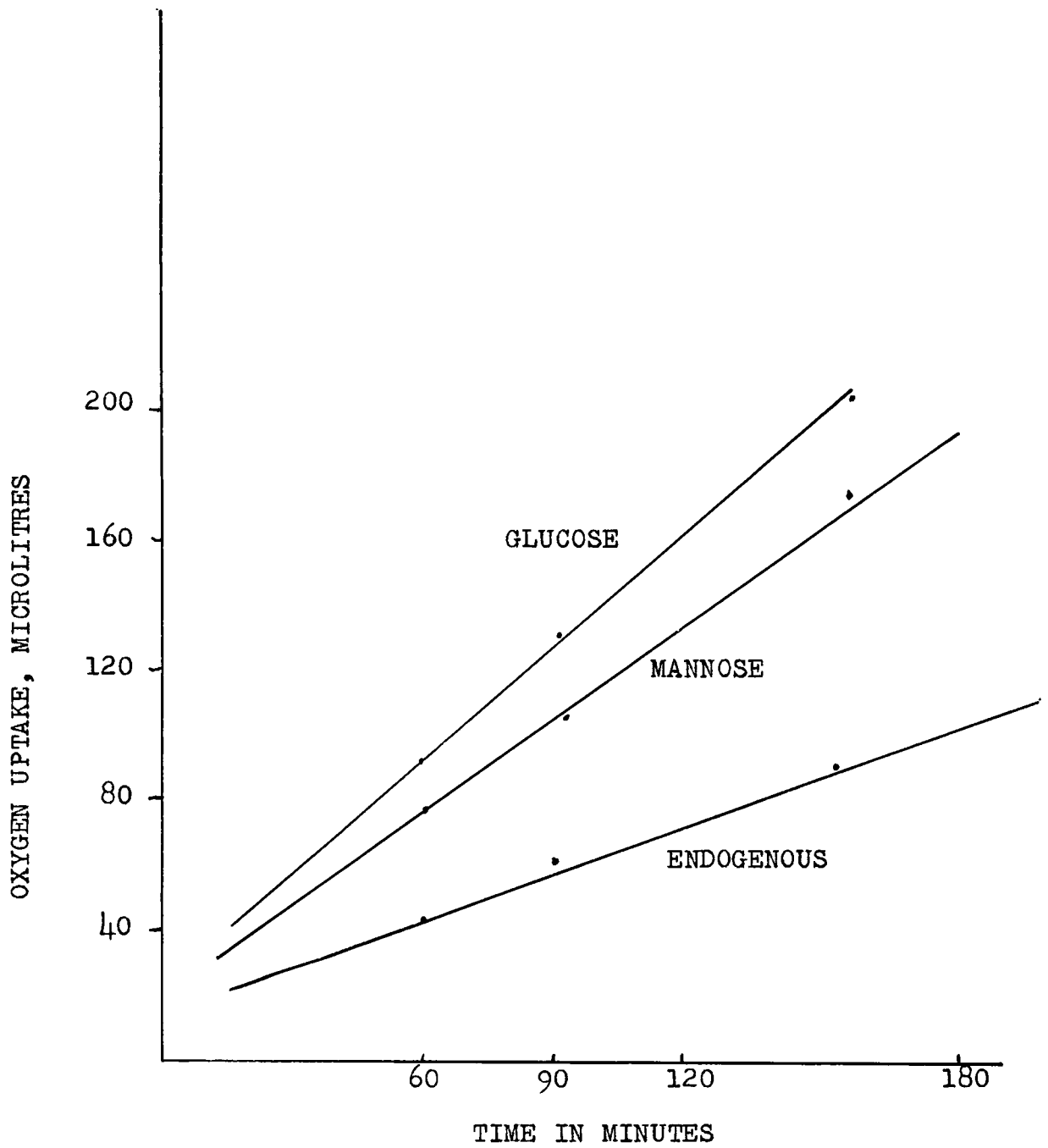


Figure 4. Effect of glucose and mannose on the oxygen uptake by *Sporocytophaga* strain 5-80.

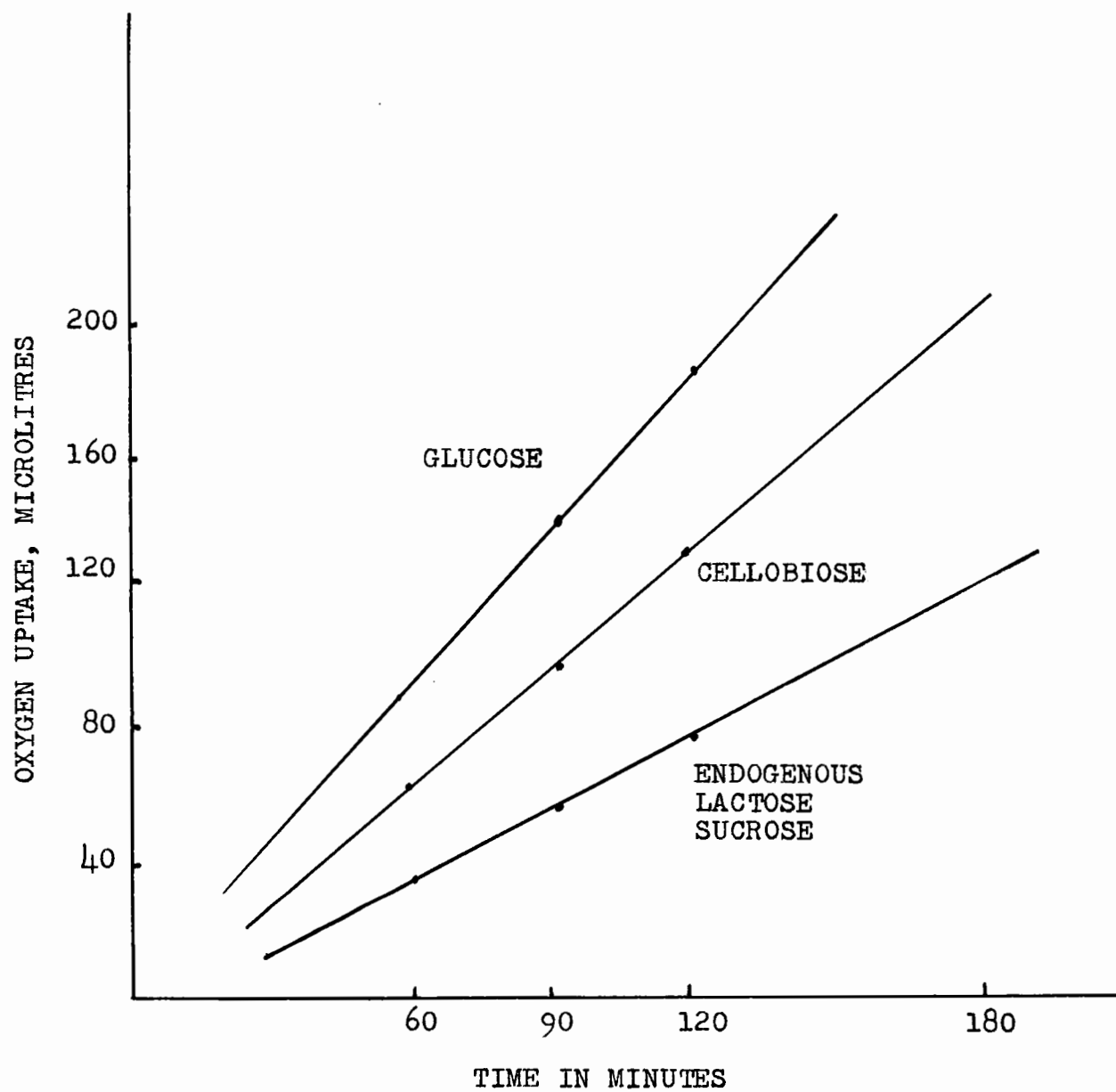


Figure 5. Effect of glucose, cellobiose, lactose and sucrose on the oxygen uptake by Sporocytophaga strain 5-80.

Table XXI

The effect of glucose concentration on oxygen uptake by Sporocytophaga strain 5-80.

Time minutes	Accumulative oxygen uptake in microlitres			
	Glucose concentration			
	18.0%	9.0%	.9%	.09%
	M/1	M/2	M/20	M/200
60	0	14.3	13.7	14.2
120	0	37.1	50.5	59.9
210	0	43.7	59.9	75.5

Table XXII

The effect of glucose concentration on oxygen uptake by Sporocytophaga strain 5-80.

Time minutes	Accumulative oxygen uptake in microlitres			
	Glucose concentration			
	0.18%	0.018%	0.0018%	0.000
	M/100	M/1000	M/10,000	
45	21.4	12.7	9.4	6.9
75	33.4	20.9	17.0	13.3
135	57.3	37.4	29.6	27.4
195	82.6	60.9	45.3	44.7

Table XXIII

The effect of glucose concentration on oxygen uptake by Sporocytophaga strain 5-80.

Accumulative oxygen uptake in microlitres

Glucose concentration

Time minutes	4.5%	0.09%	0.0009%	0.0000
	M/4	M/200	M/20,000	
60	21.5	34.0	12.6	10.4
120	52.5	83.9	32.0	31.1
180	82.5	134.4	49.9	46.1

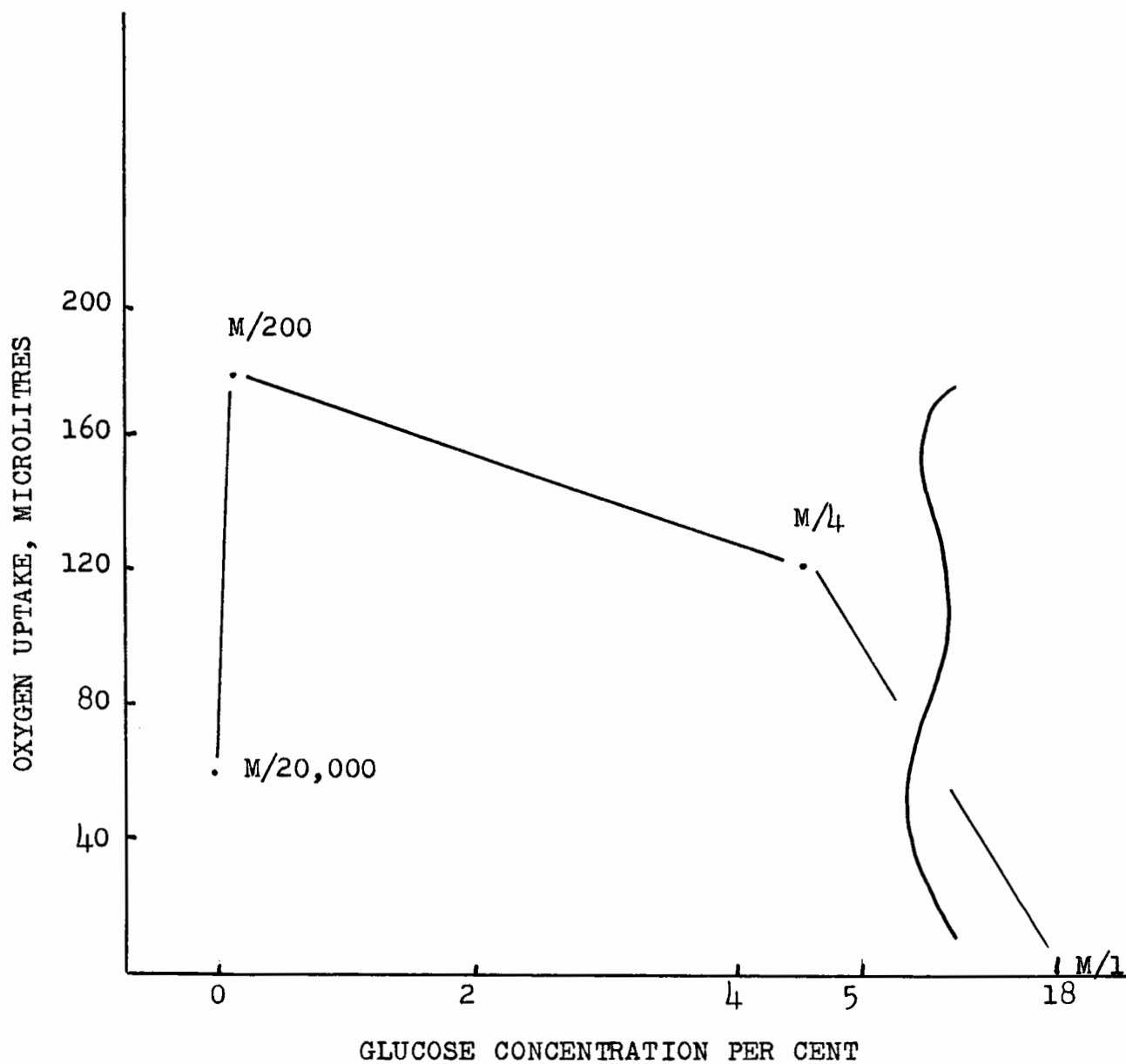


Figure 6. Effect of glucose concentration on oxygen uptake by Sporocytophaga strain 5-80.

Urea, Peptone

Urea, 0.1 per cent, and Difco proteose-peptone, 0.05 per cent, had no effect on respiration by Sporocytophaga strain 5-80 (Tables XXIV, XXV and Figure 7).

Yeast extract

Endogenous respiration of young cultures, 50 hours old, of Sporocytophaga strain 5-80, was increased by 0.05 per cent yeast extract but no effect was observed with 12 day old cultures (Table XXVI and Figure 8).

pH

Maximum glucose respiration was obtained at pH 7.2. Respiration occurred at the pH range of 5.9 to 8.5. Initial pH changed from 8.5 to 7.75, 7.2 to 7.15, 6.5 to 6.7 and 5.9 to 6.15 (Table XXVII and Figure 9).

Table XXIV

The effect of urea on oxygen uptake
by Sporocytophaga strain 5-80.

Accumulative oxygen uptake in microlitres

Time minutes	Endogenous	Urea	Glucose & urea	Glucose
60	18.0	18.5	33.8	33.0
90	27.3	25.1	52.0	50.8
120	33.8	33.9	70.6	68.6
180	46.6	46.5	101.8	97.8

Table XXV

The effect of proteose-peptone on oxygen
uptake by Sporocytophaga strain 5-80.

Accumulative oxygen uptake in microlitres

Experiment no.	Time minutes	Endogenous	Peptone	Peptone & glucose	Glucose
1	150	66.6	62.2	140.5	138.0
2	120	43.5	47.7	96.2	95.3

.05 per cent peptone

M/200 glucose

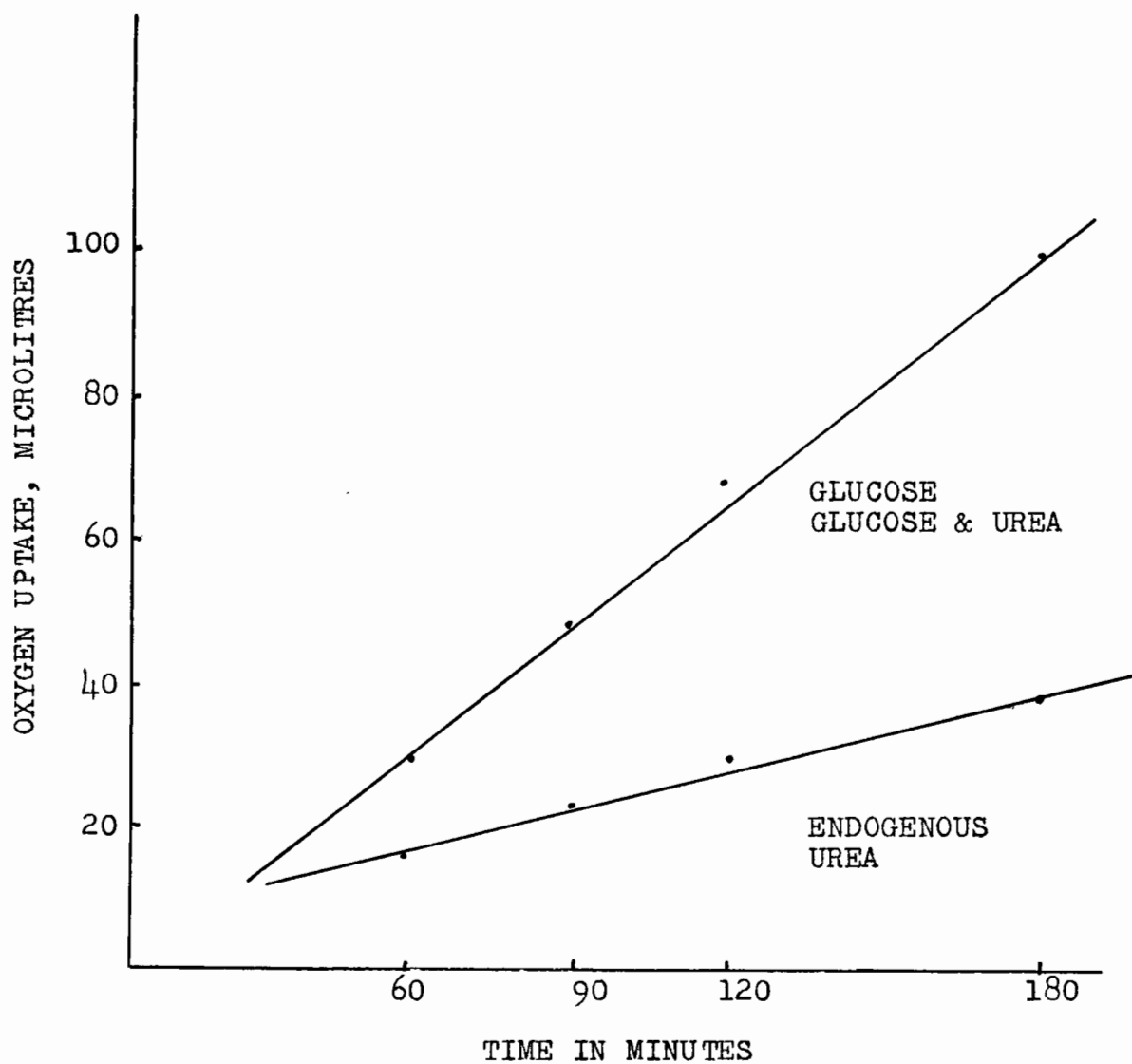


Figure 7. Effect of urea on glucose and endogenous oxygen uptake by Sporocytophaga strain 5-80.

Table XXVI

The effect of yeast extract on oxygen uptake by Sporocytophaga strain 5-80 harvested after 50 hours and 12 days.

Accumulative oxygen uptake in microlitres.

Age of culture	Time minutes	Endogenous	Yeast extract	Glucose & yeast extract	Glucose
50 hours	135	44.4	72.7	110.4	108.9
12 days	150	151.0	150.0	205.0	197.0

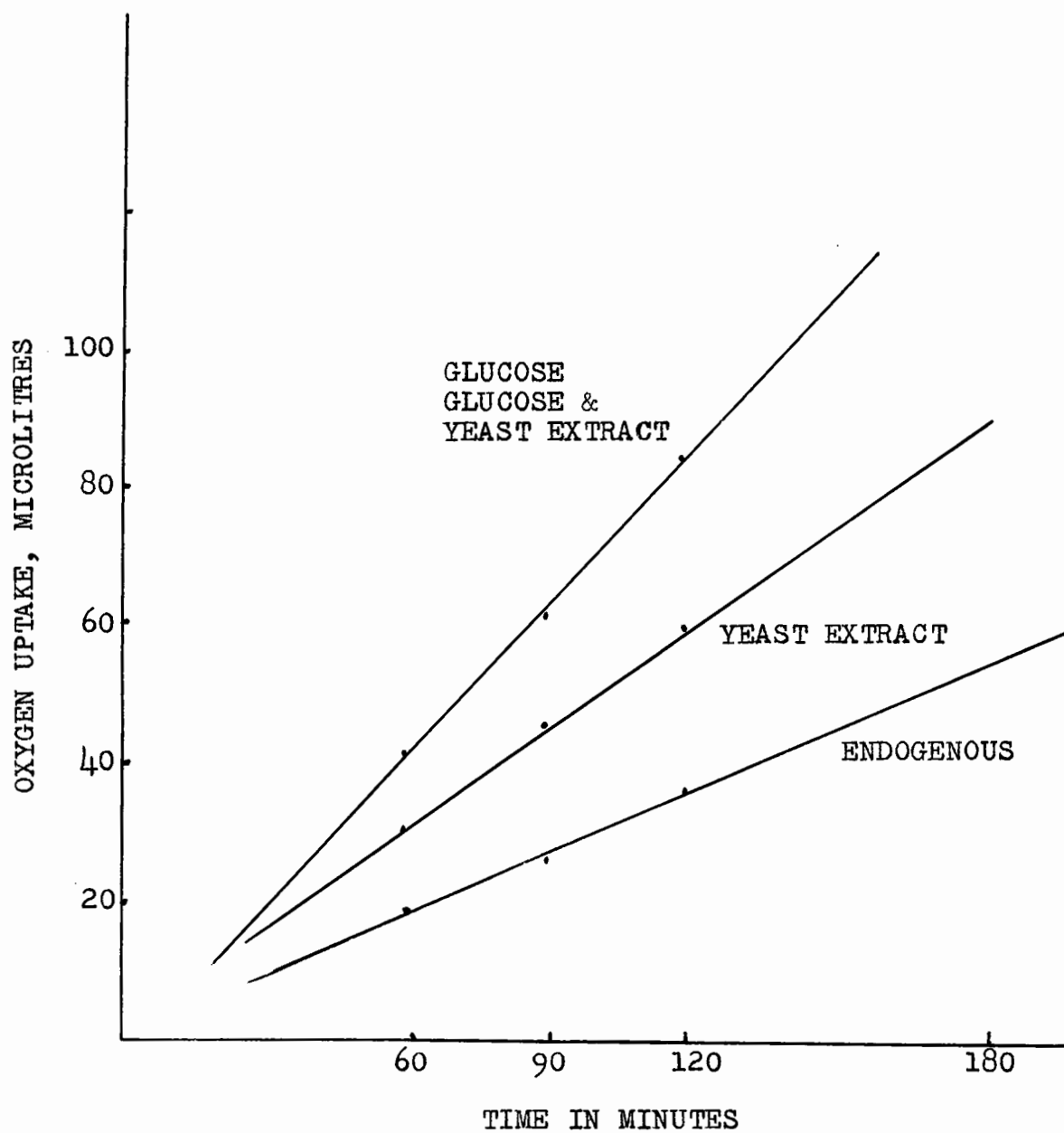


Figure 8. Effect of yeast extract on glucose and endogenous respiration by Sporocytophaga strain 5-80.

Table XXVII

Effect of pH on glucose respiration
by Sporocytophaga strain 5-80.

Accumulative oxygen uptake in microlitres

	pH range			
Initial pH	5.9	6.5	7.2	8.5
Time minutes				
60	25.2	35.3	44.5	35.1
120	35.8	56.3	77.6	67.6
165	46.3	79.2	113.8	94.2
Final pH	6.15	6.7	7.15	7.75
Per cent decrease from pH 7.2	59.2	29.5	0.0	17.1

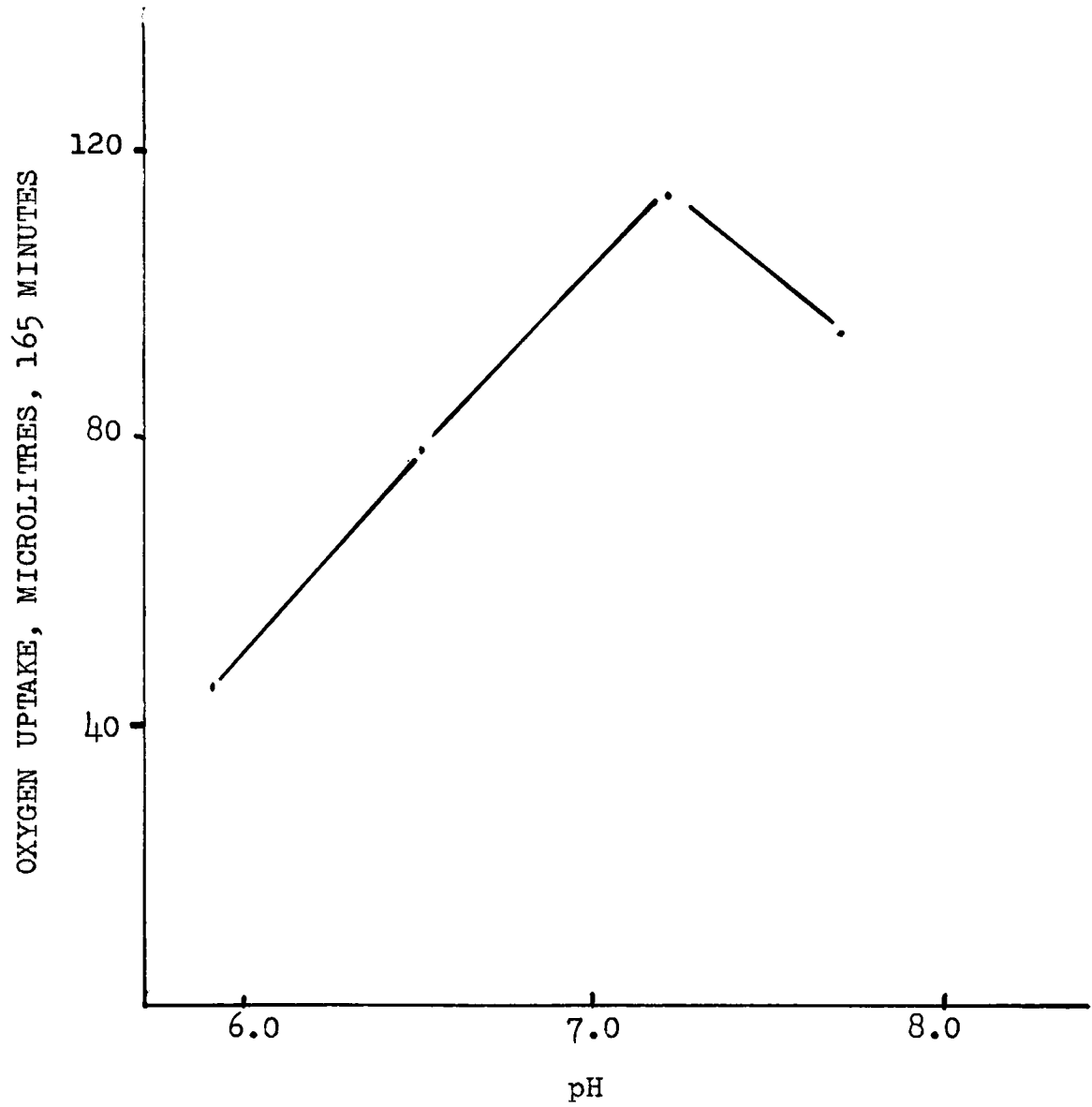


Figure 9. Effect of pH on glucose respiration by Sporocytophaga strain 5-80.

The effects of amino acids on respiration by Sporocytophaga strain 5-80 are shown in Table XXVIII and Appendix Tables XXXVII and XXXVIII. Cysteine had a pronounced inhibitory effect on glucose and endogenous respiration. M/100 cysteine inhibited endogenous respiration by 73 per cent and glucose respiration by 74 per cent. M/1,000 cysteine decreased endogenous 18.0 per cent and glucose respiration 62.0 per cent. M/10,000 cysteine had no effect on endogenous or glucose respiration (Table XXIX, Figures 10, 11); leucine and lysine inhibited glucose respiration 30.0 per cent and 37.0 per cent respectively. Histidine, tyrosine, phenylalanine had a 19.0 per cent inhibitory action on glucose respiration. Hydroxy-1-proline, 1-proline, alanine, asparagine and aspartic acid inhibited glucose respiration by 9 to 14 per cent. Tryptophan inhibited endogenous respiration 24 per cent.

Proline and 1-glutamic acid increased endogenous respiration 21 per cent and 25 per cent respectively. Taurine had a 16 per cent and d-glutamic acid a 13 per cent stimulatory effect on endogenous oxygen uptake.

Table XXVIII

The effect of amino acids on glucose and endogenous respiration by Sporocytophaga strain 5-80.

Per cent increase or decrease in respiration produced by amino acid on glucose and endogenous respiration.

	Endogenous		Glucose	
	Per cent Increase	Per cent Decrease	Per cent Increase	Per cent Decrease
Hydroxy-l-proline	-	-	-	14
l-Histidine	-	-	-	17
l-Tyrosine	-	-	-	19
l-Proline	21	-	-	11
dl-Valine	-	-	-	9
l-Taurine	16	-	-	-
dl-Phenylalanine	-	-	-	19
dl-Leucine	-	-	-	30
dl-Lysine	-	-	-	37
dl-Alanine	-	-	-	10
Asparagine	-	-	-	9
dl-Aspartic acid	-	-	-	9
l-Cysteine	-	73	-	74
d-Glutamic acid	13	-	-	-
l-Glutamic acid	25	-	-	-
dl-Tryptophan	-	24	-	-

Table XXIX

The effect of cysteine on oxygen uptake
by Sporocytophaga strain 5-80.

Accumulative oxygen uptake in microlitres.

Time minutes	Molar concentration	Per cent	Endog- enous	Cysteine	Glucose and cysteine	Glucose
240	M/100	0.024	58	16	37	139
240	M/1000	0.0024	51	42	54	99
180	M/10000	0.00024	74	78	227	216

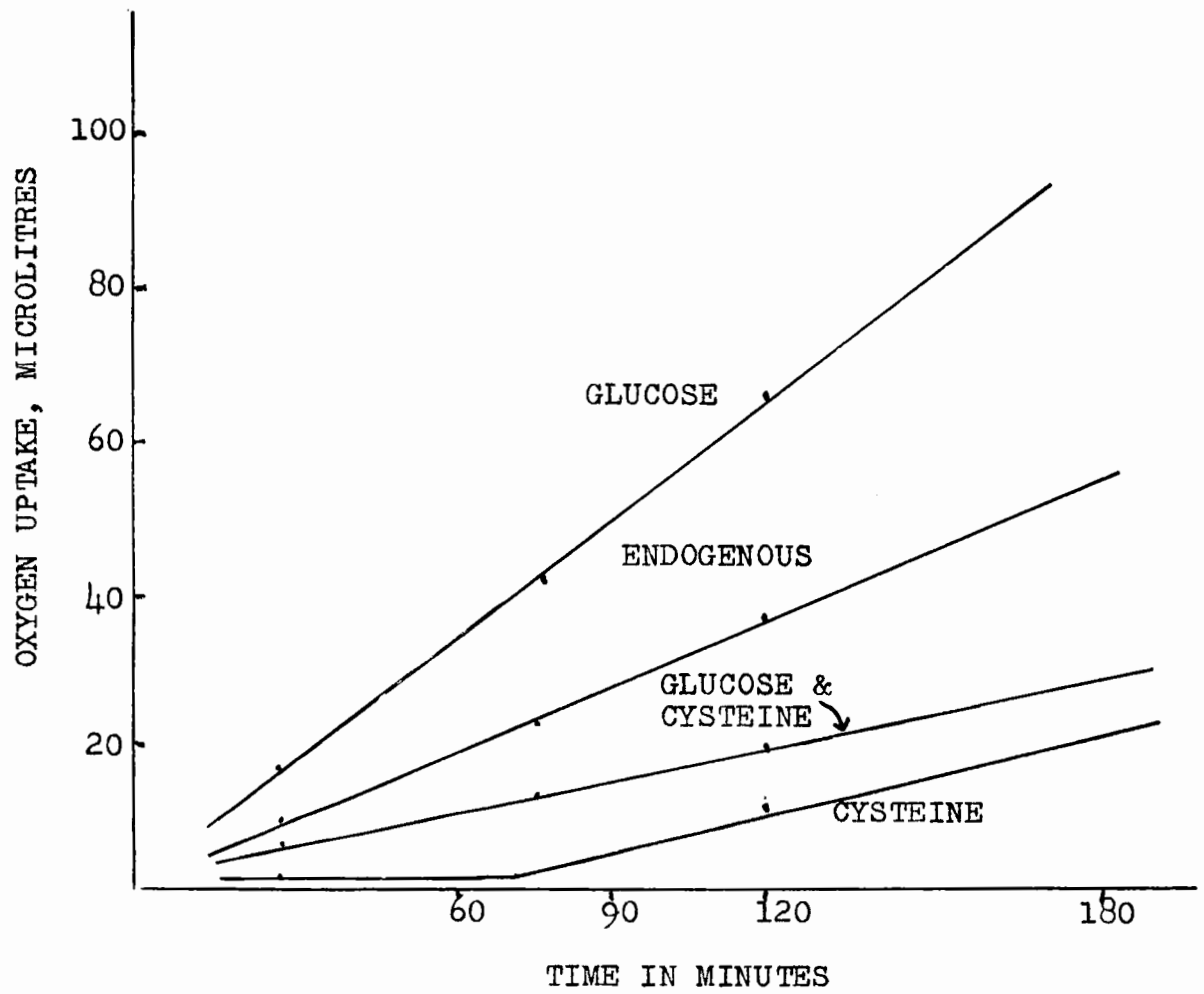


Figure 10. Effect of M/100 cysteine on glucose and endogenous respiration by Sporocytophaga strain 5-80.

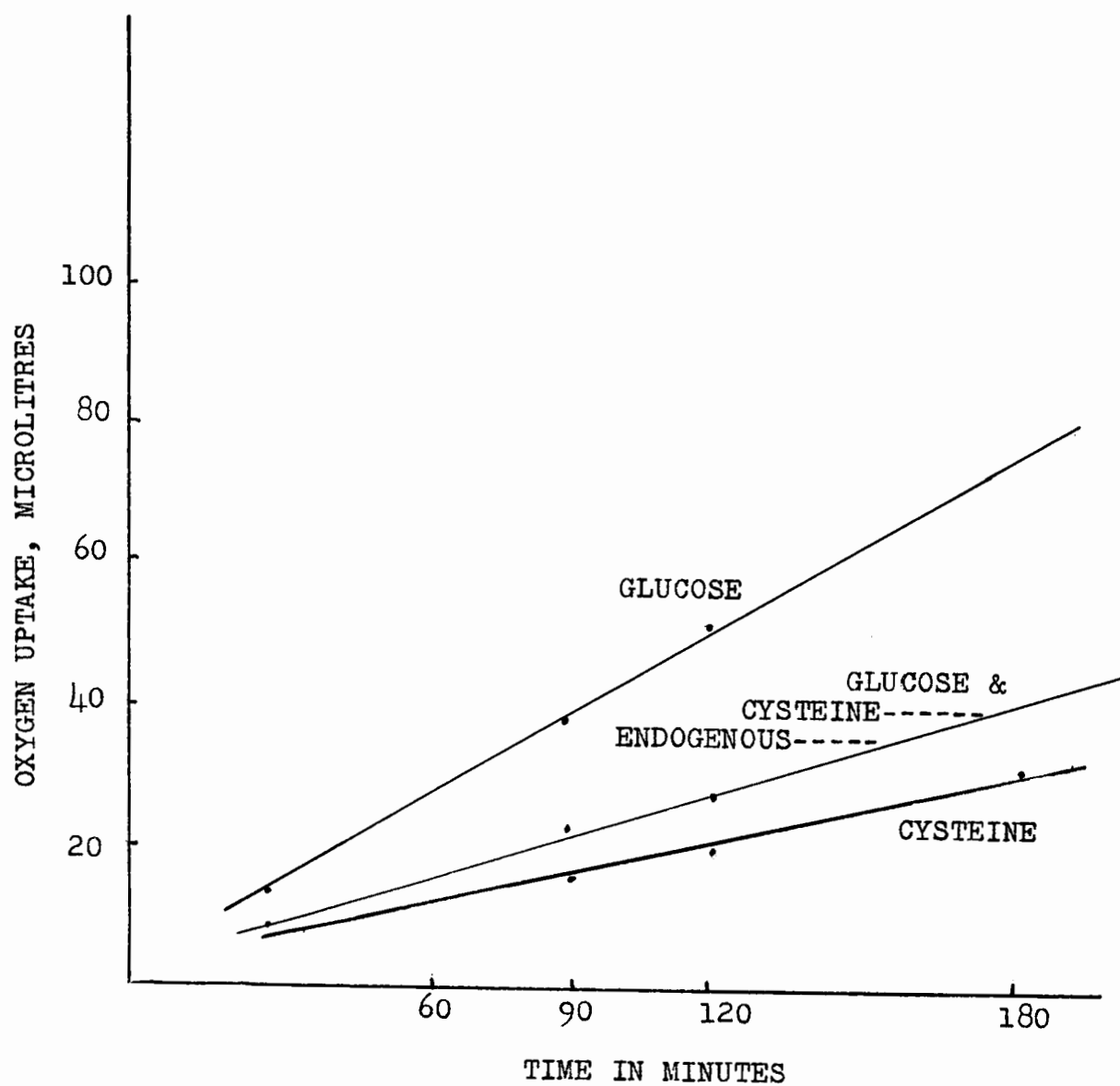


Figure 11. Effect of M/1000 cysteine on glucose and endogenous respiration by Sporocytophaga strain 5-80.

DISCUSSION

Sporocytophaga strains were isolated by the enrichment method and purified by plating on glucose agar, as suggested by Stanier (1942). Development on glucose agar, however, was sporadic. Fahraeus (1949) suggested that the irregular growth on glucose agar may be due to the presence of organic impurities which could be removed by prolonged treatment of agar with water. Washed agar, however, did not improve growth. The nature of the inhibiting agents in agar was not determined.

The relationship between the vegetative and microcyst stages was investigated. An attempt was made to obtain an amicrocystogenous strain from the American Type Culture Collection strain number 10010, by continuous subculture of a dilute inoculum of vegetative cells. The growth from a dilute inoculum was sporadic. The necessity for a large inoculum was observed by previous workers (Stanier, 1942, Fahraeus, 1947) and explained by assuming the need for an essential growth factor which was carried over in the inoculum. An amicrocystogenous strain was not obtained from the American Type Culture Collection strain number 10010 in the dilution experiment.

The metabolism of the cellulose decomposing Cytophagas

was shown to be similar to that of other microorganisms. Cellulose was decomposed to simple sugars which were used by the cell as a source of carbon and energy.

Glucose, mannose and cellobiose were the only carbohydrates utilized in cultural and respiration experiments by the isolated Sporocytophaga strains. Sijpesteijn & Fahraeus (1949) showed that growth of Sporocytophaga myxococcoides occurred readily in 0.1 per cent glucose "Whereas in corresponding media with 0.5 and 1.0 per cent glucose there was a pronounced lag in development which lasted for several weeks". They assumed that relatively high concentrations of glucose "paralyzed" the normal metabolism, for if cellulose was simultaneously present it was not attacked during the lag period. Cells became adapted to the higher glucose concentration, so that in presence of cellulose and glucose, glucose was preferentially attacked and cellulose breakdown started only when the greater part of the glucose had been consumed (Sijpesteijn & Fahraeus, 1949).

Respiration studies with strain 5-80 showed that M/200 (0.09 per cent) glucose was the optimum concentration. High glucose concentration, 4.5 per cent, inhibited glucose but not endogenous respiration, suggesting that adaptation must take place before the Cytophagas can grow in high glucose concentrations.

Ammonium and nitrate were the best sources of nitrogen for cellulose decomposition and growth in glucose medium. Cellulose decomposition was observed in all strains with asparagine, cysteine and d-glutamic acid, as sole sources of nitrogen. Endogenous oxygen uptake of strain 5-80 was stimulated by valine, l-glutamic acid, d-glutamic acid and taurine. Asparagine was the only amino acid which showed good growth comparable to that with ammonium or nitrate. When asparagine was omitted from the combination of amino acids, cellulose decomposition was either absent or strongly inhibited.

The inoculum for the experiments on the effects of amino acids on cellulose decomposition was purposely a heavy one, since preliminary studies had shown the unsuitability of amino acids as a source of nitrogen. The decomposition of filter paper may have been caused by the deposition of attacked cellulose fibres on the surface of the filter paper strip. This may explain observed growth in cysteine, lysine and tyrosine when present as a source of nitrogen.

The inhibitory effects of amino acids on respiration by strain 5-80 may be grouped as follows: (a) cysteine, 73 per cent, (b) leucine, lysine, 30 per cent, (c) phenylalanine, tyrosine, tryptophan and histidine, 19 per cent.

Visible cellulose decomposition by Sporocytophaga strain 5-80 occurred in 15 days when cysteine was omitted from the combination of amino acids, compared to 28 days in presence of all amino acids. In media containing cysteine with NaNO_3 , cellulose decomposition occurred in 10 days as compared to 3 days in control. The same concentration of cysteine, 0.025 per cent, strongly inhibited glucose and endogenous oxygen uptake; 0.0025 per cent cysteine inhibited glucose but not endogenous respiration. The effect of cysteine may be due to a lowering of the oxidation reduction potential which would be effective in a glucose liquid medium but not in cellulose basal medium where growth occurs at the air water interface.

Leucine inhibited cellulose decomposition and glucose respiration by strain 5-80 and was toxic to strain X-20 (no cellulose decomposition occurred in 28 days). Lysine had no effect on cellulose decomposition but inhibited glucose respiration of strain 5-80.

Cellulose decomposition by strain 5-80 occurred in group medium A-7, 14 amino acid combination less phenylalanine, tyrosine, tryptophan, in 7 days as compared to 28 days for the control. The inhibitory effect of these three amino acids was also demonstrated in respiration studies and may be due to a similarity in their chemical structure, namely a

benzene ring structure with an alanine side chain. Histidine with a similar chemical structure, imidazole ring and alanine side chain, also inhibited glucose respiration of strain 5-80. (Gladstone, 1939).

The 9 to 14 per cent inhibition of respiration obtained with hydroxy-l-proline, proline, alanine, asparagine and aspartic acid did not appear to be high enough to be regarded as an inhibitory effect.

Hutchinson & Clayton (1919) and Reese (1947) showed that urea had a "toxic" effect on cellulose decomposition by Sporocytophaga myxococcoides. Stanier (1942) demonstrated cellulose decomposition by Sporocytophaga myxococcoides, with urea as sole source of nitrogen, but not by Cytophaga hutchinsonii and Cytophaga rubra. The method of sterilizing urea was not stated by the two latter workers.

Endogenous and glucose respiration by Sporocytophaga strain 5-80 was not inhibited by 0.1 per cent urea. The urea was not sterilized for the respiration experiment. The inhibitory effect observed by Reese (1947) and Hutchinson & Clayton (1919) may have been due to toxic compounds produced by heat sterilization of urea.

The observed stimulation of cellulose decomposition by Cytophaga strains, in presence of associated bacteria (Harmsen, 1946) or by large inocula (Fahraeus, 1947)

was attributed to an enrichment of the basal medium with essential growth factors.

Cellulose decomposition by Sporocytophaga strain 5-80 was enhanced with yeast extract and by combinations of growth factors. The experiments indicated the necessity for using a controlled inoculum. The inoculum for the cellulose decomposition studies was a washed suspension of centrifuged glucose grown cells and mucilage. The suspension may not have been completely washed free of growth factors, since the cells could not be separated from the mucilage by centrifugation. Respiration studies showed the importance of the condition of the cell suspension. Stimulation of endogenous respiration was observed only with washed 50 hour old suspensions of strain 5-80.

Results from cultural experiments are difficult to analyze because of the inoculum factor. Sporocytophaga cells grow in contact with cellulose fibres or form a heavy mucilage in glucose broth. A cell suspension could not easily be obtained from agar surfaces because (1) growth occurred only when large inocula were used; (2) a weak agar gel was required (1.0 per cent) making harvesting difficult, and (3) growth on glucose agar was sporadic. An attempt was made to grow mass cultures, in aerated

glucose yeast extract broth and to separate the cells with the continuous flow Sharples supercentrifuge.

The method was cumbersome and yielded a cell suspension with a high mucilage content. The technique finally adopted, namely, candle filtration (page 63) was simple, sterile conditions could be easily maintained throughout, harvesting time could be adjusted to the growth rate of the organism and most important, active cell suspensions were obtained relatively free of mucilage.

The Warburg method showed clearly and quantitatively the effect of organic compounds on the respiration of Sporocytophaga.

SUMMARY AND CONCLUSIONS

Sporocytophaga strains were isolated from soil and manure and the bacteria were purified by plating enriched cultures on glucose agar.

The effects of nitrogen compounds, carbohydrates and growth factors, on the metabolism of the isolated strains were studied.

Glucose, mannose and cellobiose were the only carbohydrates utilized by the cellulose decomposing Sporocytophaga strains. High concentrations 4.5 per cent, M/4 of glucose inhibited glucose but not endogenous respiration. The optimum glucose concentration for strain 5-80 was M/200.

Ammonium and nitrate were preferred sources of nitrogen. Glycine, leucine and lysine, greatly inhibited glucose respiration. The inhibition caused by phenylalanine, tryptophan, tyrosine and histidine, may be due to a similarity in chemical structure.

Respiration by strain 5-80 was not inhibited by urea or by proteose-peptone.

Yeast extract stimulated cellulose decomposition and endogenous respiration of strain 5-80.

An active cell suspension for respiration studies was obtained by harvesting the cells from the surface of candle filters.

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APPENDIX

REGENERATED CELLULOSE

Kalnins' modification of Scales method (Kalnins, 1930)

1. 10 grams of finely cut filter paper, Whatman No. 41 or Whatman purified cellulose powder was dissolved for not more than 20 seconds in 72 per cent H_2SO_4 at 60 to 65°C.
2. Cellulose was reprecipitated by bringing the volume rapidly up to 2 litres with ice water. The precipitated cellulose was allowed to settle overnight in 10 to 15 litres of tap water.
3. Supernatant liquid was siphoned off and the precipitate filtered in a large Büchner funnel with repeated washings until all traces of acid were removed.
4. The cellulose mat obtained by filtration was resuspended in 100 ml of water, for storage.
5. An even, milky suspension was obtained with the Waring blender.
6. The concentration of cellulose was calculated from the difference in weight of 25 ml of the cellulose suspension and 25 ml of water.
7. Cellulose was added to basal medium at a concentration of 0.4 per cent.

Water insoluble cellulose dextrin. Cold sulphuric acid method (Fuller & Norman, 1942)

1. Mortar, pestle and 100 ml H_2SO_4 (72 per cent) were chilled in the refrigerator for 2 to 3 hours.
2. Twenty grams of finely cut filter paper were slowly added to acid; to prevent a rise in temperature, the mortar was surrounded with water containing generous quantities of cracked ice. The temperature was not allowed to rise above 10 to 12°C.
3. The mixture was kneaded carefully until the cellulose was fully dispersed.
4. The solution was allowed to stand in the water for 1 to 1½ hours.
5. A water insoluble cellulose dextrin was obtained by pouring the acid solution into approximately 600 ml of ice water containing pieces of ice.
6. The precipitated dextrin was filtered through several layers of cheesecloth over a large Büchner funnel
7. The precipitate was resuspended, washed several times to remove the acids and then neutralized with dilute alkali, after which the precipitate was again washed to remove all soluble salts.

Schweitzer's solution

1. 10 grams of filter paper were placed in a tight stoppered bottle with 100 cc of Schweitzer's solution. (Slight excess of CuCO_3 added to 1 litre of dilute NH_4OH solution, allowed to stand overnight. The supernatant solution was siphoned off).
2. The bottle was shaken for 1 hour on a shaker.
3. Pulp was poured out into about 1 litre of distilled water.
4. Excess of 10 per cent HCl was added.
5. The precipitated cellulose was washed on a Büchner funnel till free of chloride.
6. Washed pulp was suspended in 100 ml of water.
7. Concentration was determined and the cellulose was added to basal salts medium to a final concentration of 0.4 per cent.

Appendix Table XXX

Growth of Sporocytophaga strains in cellulose basal medium A in presence of growth factors

Sporocytophaga strains

		X-20				5-80				XXB				B-1			
		Time in days and degree of cellulose decomposition															
Growth factor	medium	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24
Series 1	a	-	-+	++	++	-	-+	++	++	+	++	+++	+++	-+	+	++	+++
	b	-	-+	++	++	-	-+	+	++	+	++	+++	+++	-	-+	+	+++
	c	-	+	++	++	-	-+	++	++	+	++	+++	+++	-+	-+	++	+++
	d	-	-+	++	++	-+	-+	++	+++	+	++	+++	+++	-+	-+	+	+++
	e	-	-+	++	++	-+	-+	++	+++	+	++	+++	+++	-+	-+	-+	++
Series 2	a	-	-+	++	++	-+	+	++	+++	+	++	+++	+++	-	-+	+	+++
	b	-	-+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	c	-	-+	+	+++	-+	-+	++	+++	+	++	+++	+++	-+	+	++	+++
	d	-	-+	++	++	-+	-+	++	++	+	++	+++	+++	-+	-+	+	++
	e	-	-+	+	++	-+	-+	++	+++	-+	+-	+	+	-+	-+	++	+++
Control		-	+	++	+++	-	+	++	+++	+	++	+++	+++	-+	+	++	+++

Appendix Table XXXI

Growth of Sporocytophaga strains in cellulose basal medium A in presence of growth factors

Sporocytophaga strains

		X-20				5-80				XXB				B-1			
		Time in days and degree of cellulose decomposition															
Growth factor	medium	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24
Series 3	a	-	-	++	+++	-+	+	++	+++	+	++	++	++	-+	+	++	+++
	b	-	-+	++	+++	-+	+	++	++	+	++	++	++	-+	+	++	+++
	c	-	-+	++	+++	-+	+	++	+++	+	++	++	++	-+	+	++	+++
	d	-	-	++	++	-+	+	++	++	+	++	++	++	-+	+	++	+++
	e	-	-+	++	++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
Series 4	a	-+	+	++	+++	-+	+	++	+++	+	++	+++	+++	-+	+	++	+++
	b	-+	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	c	-+	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	d	-+	+	+	++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	e	-+	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
Control		-	+	++	+++	-	+	++	+++	+	++	+++	+++	-+	+	++	+++

Appendix Table XXXII

Growth of Sporocytophaga strains in cellulose basal medium A in presence of growth factors

Sporocytophaga strains

		X-20				5-80				XXB				B-1			
		Time in days and degree of cellulose decomposition															
Growth factor medium		4-5	9	14	24	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24
Series 5	a	-+	+	++	+++	-+	+	++	++	+	++	+++	+++	+	+	++	++++
	b	-+	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	+	+++
	c	-	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	+	+++
	d	-	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	+	++
	e	-	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	+	+++
Series 6	a	-	-+	++	+++	-+	+	++	++	+	+	+++	+++	-+	+	++	+++
	b	-	-+	++	+++	-+	+	++	++	+	+	+++	+++	-+	+	++	+++
	c	-	-+	++	+++	-+	+	++	++	+	+	+++	+++	-+	+	++	+++
	d	-	-+	++	+++	-+	+	++	++	+	+	+++	+++	-+	+	++	+++
	e	-	-+	++	+++	-+	+	++	++	-+	+	+++	+++	-+	+	++	+++
Control		-	-+	++	+++	-	+	++	+++	+	++	+++	+++	-+	+	++	+++

Appendix Table XXXIII

Growth of Sporocytophaga strains in cellulose basal medium A in presence of growth factors

Sporocytophaga strains

		X-20				5-80				XXB				B-1			
		Time in days and degree of cellulose decomposition															
Growth factor	medium	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24
Series 7	a	-	-	-	+	-	-	-	+	-+	++	+++	+++	-	-	-	+
	b	-	-	-	+	-	-+	++	+++	+	++	+++	+++	-	-	-	+
	c	-	-	-+	+++	-	-+	++	+++	+	++	+++	+++	-+	+	++	++
Series 8	a	-	-	-	+	-	-	-	+	-	+	++	++	-	-+	+	++
	b	-	-	-	+	-	-+	+	+	-+	+	++	+++	-	-+	++	++
	c	-	-	-	+	-	-	-	+	-	-	++	++	-	-	-	-
Series 9	a	-	-	-	+	-	-	-+	++	-+	+	++	+++	-	-	+	+++
	b	-	-	-	+	-	-	+	++	-+	+	++	+++	-	-+	++	+++
	c	-	-	-	+	-	-	+	++	-+	+	++	+++	-	-+	+	+++
Control		-	+	++	+++	-	+	++	+++	+	++	+++	+++	-+	+	++	+++

Appendix Table XXXIV

Growth of Sporocytophaga strains in cellulose basal medium A in presence of growth factors

Sporocytophaga strains

		X-20				5-80				XXB				B-1			
		Time in days and degree of cellulose decomposition															
Growth factor	medium	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24
Series 10	a	-	-+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	b	-	-+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	c	-	-	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
Series 11	a	-	-+	+	+	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	b	-	+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	c	-	-+	++	++	-+	+	+	+	+	++	+++	+++	-+	+	++	+++
Series 12	a	-	-+	++	+++	-+	+	++	++	+	++	+++	+++	-+	+	++	+++
	b	-	-+	++	+++	-+	+	+	+	+	++	+++	+++	-+	+	++	+++
	c	-	-+	++	++	-+	+	+	++	+	++	+++	+++	-+	+	++	+++
Control		-	+	++	+++	-	+	++	+++	+	++	+++	+++	-+	+	++	+++

Appendix Table XXXV

Growth of Sporocytophaga strains in cellulose
basal medium A in presence of growth factors

Sporocytophaga strains

		X-20				5-80				XXB				B-1			
		Time in days and degree of cellulose decomposition															
Growth factor	medium	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24
Series 13	a	-	-+	++	+++	-+	+	+	++	+	++	+++	+++	-+	+	++	+++
	b	-	-	++	+++	-+	++	++	+++	+	++	+++	+++	-+	+	++	+++
	c	-	-	-	+	-+	+	+	+++	+	++	+++	+++	-+	+	++	+++
Series 14	a	-	+	++	+++	-+	+	+	+++	+	++	+++	+++	-+	+	++	+++
	b	-	-	++	+++	-+	+	+	+++	+	++	+++	+++	-+	+	++	+++
	c	-	+	++	+++	-+	+	+	++	+	++	+++	+++	-+	+	++	+++
Series 15	a	-	-+	++	+++	-+	+	+	+++	+	++	+++	+++	-+	+	++	+++
	b	-	+	++	+++	-+	+	+	+++	+	++	+++	+++	-+	+	++	+++
	c	-	-	++	+++	-+	+	+	+	+	++	+++	+++	-+	+	++	+++
Control		-	+	++	+++	-	+	++	+++	+	++	+++	+++	-+	+	++	+++

Appendix Table XXXVI

Growth of Sporocytophaga strains in cellulose basal medium A in presence of growth factors

Sporocytophaga strains

	X-20				5-80				XXB				B-1			
	Time in days and degree of cellulose decomposition															
Growth factor medium	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24	4-5	9	14	24
Series 16	-	+	++	+++	-	+	+	++	+	++	+++	+++	-+	+	++	+++
Series 17	-	-	-	+	-	-	-	++	-	+	++	+++	-	+	++	+++
Series 18	-	-	-	+	-	-	-	+	-	+	++	++	-	-	+	+++
Series 19	-	-	-	+	-	+	+	++	-	+	+	++	-	+	++	+++
Series 20	-	-	++	+++	-+	+	++	+++	+	++	+++	+++	-+	+	++	+++
Series 21	-	-+	++	+++	-+	+	++	+++	+	++	+++	+++	-+	+	++	+++
Control	-	+	++	+++	-	+	++	+++	+	++	+++	+++	-+	+	++	+++

Appendix Table XXXVII

The effect of amino acids on oxygen uptake by Sporocytophaga strain 5-80

Accumulative oxygen uptake in microlitres in 180 minutes

Amino acid	Molar conc. 10 ⁻³	Endo- gen- ous	Amino acid	Glu- cose & amino acid	Glu- cose
Hydroxy-l-proline Amino acid & glucose	1.9	42.5 50.4	44.1 -	- 84.0	81.6 98.1
dl-Ornithine Amino acid & glucose	1.9	42.5 50.4	43.7 -	- 98.5	81.6 98.1
l-Histidine Amino acid & glucose	1.6	42.5 50.4	38.8 -	- 79.1	81.6 98.1
l-Tyrosine Amino acid & glucose	1.4	52.9 -	47.5 -	- 109.5	127.2 135.2
l-Proline Amino acid & glucose	2.1	52.9 -	64.3 -	- 120.5	127.2 135.2
dl-Valine Amino acid & glucose	2.1	52.9 -	56.7 -	- 123.3	127.2 135.2
l-Arginine Amino acid & glucose	1.4	47.6 38.4	49.9 -	- 75.7	82.8 71.3
dl-Threonine Amino acid & glucose	2.0	47.6 38.4	41.6 -	- 78.6	82.8 71.3

Appendix Table XXXVII (con't)

The effect of amino acids on oxygen uptake by Sporocytophaga strain 5-80

Accumulative oxygen uptake in microlitres in 180 minutes

Amino acid	Molar conc. 10 ⁻³	Endo- gen- ous	Amino acid	Glu- cose & amino acid	Glu- cose
dl-Serine	2.4	47.6	52.8		82.8
Amino acid & glucose		38.4	-	74.7	71.3
dl-Isoleucine	1.9	51.5	53.8	-	103.2
Amino acid & glucose		-	-	127.5	125.1
dl-Norleucine	1.9	51.5	54.2		103.2
Amino acid & glucose		-	-	126.0	125.1
l-Taurine	2.0	51.5	60.1	-	103.2
Amino acid & glucose		-	-	124.9	125.1
dl-Phenylalanine	1.5	73.1	70.4	-	112.8
Amino acid & glucose		62.5	-	121.7	150.3
dl-Leucine	1.9	73.1	66.9	-	112.8
Amino acid & glucose		62.5	-	105.5	150.3
dl-Lysine	1.7	73.1	70.7	-	112.8
Amino acid & glucose		62.5	-	95.3	150.3

Appendix Table XXXVIII

The effect of amino acids on oxygen uptake by Sporocytophaga strain 5-80

Accumulative oxygen uptake in microlitres

Amino acid	Time	Molar conc. 10 ⁻³	Endogenous	Amino acid	Glucose & amino acid	Glucose
dl-Alanine	180	2.8	53.4	51.8	113	125
Asparagine	180	1.9	123.8	125.0	218.4	238.7
dl-Aspartic acid	300	1.9	65.0	64.6	180.4	166.0
l-Cysteine	240	2.0	58.5	16.5	37.6	145.5
d-Glutamic acid	270	1.7	73.0	83.1	206.7	190.0
l-Glutamic acid	240	1.7	68.5	85.1	147.3	147.7
Glycine	150	3.3	87.6	92.7	188.7	194.5
dl-Tryptophan	195	1.2	79.1	60.5	118.7	116.6
dl-Methionine	180	1.7	64.0	66.8	124.2	122.0

